Modulation of Indole Alkaloid Composition in *Vinca minor*

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina zu Braunschweig

zur Erlangung des Grades

einer Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

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eingereicht am:	04.06.2018
mündliche Prüfung (Disputation) am:	27.07.2018

Druckjahr 2018

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikationen:

Abouzeid S., Beutling U., Surup F., Abdel Bar F.M., Amer M. M., Badria F. A., Yahyazadeh, M., Brönstrup M., Selmar D. (2017). Treatment of *Vinca minor* leaves with methyl jasmonate extensively alters the pattern and composition of indole alkaloids. *Journal of natural product*, 80;11: 2905–2909.

Selmar D., Kleinwächter M., **Abouzeid S**., Yahyazadeh M., Nowak M. (2017). The impact of drought stress on the quality of spice and medicinal plants. In: Ghorbanpour M., Varma A., (eds). *Medicinal Plants and Environmental Challenges*. Cham: Springer International Publishing, 159–175.

Yahyazadeh M., Meinen R., Hansch R., **Abouzeid S.,** Selmar D. (2018). Impact of drought and salt stress on the biosynthesis of alkaloids in *Chelidonium majus* L. *Phytochemistry*, 18;152: 204-212.

Selmar D., **Abouzeid S.**, Radwan A., Hijazin T., Yahyazadeh M., Lewerenz L., Nowak M. (2018). Horizontal Natural Product Transfer and Allelopathy: Novel insights in a so far unconsidered exchange of natural products. *Frontiers in Ecology and Evolution, section Chemical Ecology*. Accepted.

Hijazin T., Radwan A., **Abouzeid S.**, Dräger G., Selmar D. (2018). Horizontal natural product transfer – the story continues: Uptake and modification of umbelliferone. *Phytochemistry*. Submitted.

Dedicated to:

My country Egypt, *Soul of my father, my great mother, my brother, my sisters, my kind husband and my kids* (Abdel Rahman – Mennaalla).

Acknowledgments

In the name of **Allah,** the most Merciful and Beneficent.

First and Foremost, praise is to **ALLAH**, the Almighty, the greatest of all, on whom ultimately, we depend for sustenance and guidance. I would like to thank Almighty **Allah** for giving me the opportunity, determination, and strength to do my research. His continuous grace and mercy was with me throughout my life and even more during the tenure of my research.

Now, I would like to thank and express my deep and sincere gratitude to my supervisor **Prof. Dr. Dirk Selmar**, *Head of Dep. Applied Plant Biology, Institute for Plant Biology, TU Braunschweig,* for giving me the opportunity to join his lab. I appreciate all his contributions of time and ideas to make my Ph.D. experience creative and professional. Moreover, his understanding, encouraging and personal guidance have provided a good basis for the present dissertation.

I am also very appreciative of my Egyptian supervisors, **Prof. Dr. Farid A. Badria**, **Prof. Dr. Mohamed M. Amer**, and **Prof. Dr. Fatma M. Abdel Bar**, *Dep. Pharmacognosy, Faculty of Pharmacy, Mansoura University, Egypt,* for their great support and for giving me the opportunity to accomplish the Ph.D. degree in Germany.

I am very grateful to **Prof. Dr. Peter Winterhalter** and **Prof. Dr. Ludger Beerhues** of the examination committee for devoting some of their time to read and evaluate this study.

I am heartily thankful for **Prof. Dr. Mark Brönstrup** *Head of Dep. Chemical Biology, Helmholtz Centre for Infection Research,* and co-worker **Dr. Ulrike Beutling**, for the helpful advice, performing the HR-ESI-MS and LC-ESI-MS/MS experiments, answering my many questions with patience and for the helpful discussions with mass spectrometry.

I also appreciate the time and efforts of **Dr. Frank Surup,** *Dep. Microbial Drugs, Helmholtz Centre for Infection Research,* in confirming the identity of the isolated compounds by NMR.

I would like to express my thanks to **Dr. Alzahraa Radwan**, and **Dr. Mahdi Yahyazadeh** for guiding me in the first steps in the group.

I cannot forget to express my appreciation to all my colleagues and the entire members of Prof. Selmar group, **Dr. Alzahraa**, **Dr**. **Mahdi**, **Dr. Melanie**, **Mrs. Tahani**, **Ms. Carina**, and **Mrs. Sabine**. I want to thank all for the great willingness to help and the friendly atmosphere.

I wish to extend my gratitude to the Egyptian Government, **Ministry of Higher Education**, represented by the cultural affairs and missions sector in the Egyptian Embassy in Berlin for financial support. I am also grateful to the **Gleichstellungsbüro der Technischen Universität Braunschweig** for the financial support of the last six months.

I would like to extend my thanks to the head of the Department of Pharmacognosy, Mansoura University, Egypt and all my colleagues at the department for giving me the opportunity to accomplish the Ph.D. degree in Germany.

I would like to thank all my Egyptian friends for supporting me and my family to enjoy our stay in Braunschweig. Moreover, I would like to express special thanks to **Dr. Nasser Hemdan**, for the valuable discussion and the advice for organizing my thesis in a scientific way.

I owe everything to my family who encouraged and helped me at every stage of my personal and academic life and longed to see this achievement come true. I dedicate this work to my loving mother, my generous father, my sincere husband **(Mohamed)**, my daughter (**Mennaalla**), my son **(Abdelrahman)**, my sisters, and my brother. They paved my way and provided an excellent atmosphere so that I can totally concentrate on my work. Thank you all for being in my life. I love you all.

Sara Abouzeid

Braunschweig, Germany, in June 2018

Abstract

Changes in the content and the composition of natural compounds always results from the balance of their biosynthesis, allocation, and degradation. These three processes could be influenced differentially by various developmental and environmental factors. In consequence, the overall impact of these influences on the spectrum and the content of the secondary metabolites might be very complex and multifarious. However, these considerations open novel doors for manifold and deliberate approaches to change and adapt the secondary metabolism, intended to improve and optimize the search for novel pharmacologically active compounds.

With respect to environmental factors, the generation of various stress situations and the concomitant consideration of the developmental stage of the plant seems to be the most promising approaches. Since these conjunctures are governed by various signal transduction chains, the application of corresponding growth regulators or signal transducers should augment the capabilities for plant chemodiversity and phytochemical drug discovery. In this context, the most promising candidates are methyl jasmonate (MeJA), hydrogen peroxide (H_2O_2) and salicylic acid (SA), which are part of several signal transduction chains.

This thesis was aimed to comprehend the effects of MeJA, H_2O_2 and SA on the pattern of monoterpenoid indole alkaloids (MIAs) in *Vinca minor* with the special emphasis on the appearance of the novel, so far unknown indole alkaloids. The intention was to investigate whether or not the application of growth regulators could be the basis for a novel strategy to modify the composition of active components in medicinal plants.

To establish a solid basis for the identification of new, stress-induced alkaloids; firstly, the spectrum of MIAs of healthy, mature *V. minor* plants was recorded. For this, the MIAs were extracted from healthy leaves and fractionated by preparative HPLC. By means of HRMS and NMR, the main alkaloids were identified as vincamine, strictamine, 10-hydroxycathofoline, and vincadifformine.

Treatment with MeJA and H_2O_2 extensively changed the pattern and composition of the indole alkaloids present in *V. minor* plants. While 10-hydroxycathofoline and strictamine concentrations remained unaltered, the contents of vincamine and vincadifformine were dramatically reduced. Moreover, treatment with both growth regulators resulted in the accumulation of four other MIAs, identified as minovincinine, minovincine, 9-methoxyvincamine and finally a related alkaloid thought to be kopsinine. Whereas minovincinine and minovincine are already known to occur - however only in trace amounts - in *V. minor*, 9-methoxyvincamine represents a novel natural product.

Based on the high similarities of vincamine and 9-methoxyvincamine and their inverse changes in concentrations, it was postulated that vincamine was transformed to 9-methoxyvincamine. Similarly, vincadifformine was thought to be converted first to minovincinine and finally to minovincine. These modifications require oxidative enzymes. Considering the corresponding enzymes involved in the MIAs biosynthesis in *Catharanthus roseus*, it seems to be very likely that these reactions are catalyzed by cytochrome P450 enzymes. In order to verify this assumption, in parallel to treatment with MeJA, also specific enzyme inhibitors, which are known to suppress or to modulate the alkaloid biosynthesis in *C. roseus*, have been applied to the *V. minor* plants. As expected, the application of resveratrol or naproxen respectively, as oxygenases enzyme inhibitors, prevented the conversion of vincamine to 9-methoxyvincamine as well as that of vincadifformine via minovincinine to minovincine. Accordingly, these results vividly confirmed the postulated stress-induced oxidative conversion of both major MIAs in *V. minor* plants, i.e., vincamine and vincadifformine, to 9-methoxyvincamine and to minovincinine and minovincine, respectively.

The stress-induced conversions, and especially the discovery of 9-methoxyvincamine as a novel natural product, insistently outlines that even well-known, extensively studied plants such as *V. minor*, might be an auspicious source for the discovery of novel phytochemicals. Accordingly, in the future, the stress-induced changes in natural products conditions, preferably induced by the application of MeJA and other growth regulators have to be integrated into the modern search for bioactive compounds. Such approach might open new doors to improve and facilitate phytochemical drug discovery. In addition to the application of growth regulators or signal transducers, respectively, also the application of enzyme inhibitors could be used as an alternative approach to deliberately modify the content and composition of biologically active compounds in plants.

Moreover, treatments combining enzyme inhibitors and growth regulators or signal transducers might be an effective tool to study the cytochrome P450 enzymes responsible for the stress-induced conversion of MIAs. In further studies, this attractive topic should be followed enthusiastically.

The application of growth regulators or signal transducers, respectively, could modulate the composition of secondary metabolites. In combination with the dereplication-guided isolation of the induced compounds, this approach could indeed promote the discovery of novel drugs, even in well-investigated medicinal plants.

Zusammenfassung:

Veränderung des Indolalkaloid-Spektrums von *Vinca minor*

Änderungen im Gehalt von Naturstoffen und deren Zusammensetzung resultieren stets aus entsprechenden Veränderungen der Biosynthese, der Verlagerung und dem Abbau dieser Substanzen. Dabei werden diese Prozesse in unterschiedlicher Weise sowohl von Umweltfaktoren als auch von der Entwicklung und dem Wachstum der jeweiligen Pflanze beeinflusst. Hieraus folgt, dass der Einfluss all dieser Faktoren auf den Sekundärstoffwechsel sehr komplex und vielfältig ist. Dies wiederum eröffnet viele Möglichkeiten, in vielfältiger Weise absichtsvoll Änderungen des Sekundärstoffwechsels zu induzieren, um so die Suche nach neuen, pharmakologisch aktiven Naturstoffen zu verbessern.

In Hinblick auf den Einfluss unterschiedlicher Umweltfaktoren sind besonders die stress-induzierten Veränderungen vielversprechend; allerdings muss in diesem Zusammenhang stets auch der Einfluss von Wachstum und Entwicklung mit berücksichtigt werden. Entsprechend ergeben sich aus dem Einsatz verschiedener Wachstumsfaktoren und Signalsubstanzen viele unterschiedliche Möglichkeiten, den Sekundärstoffwechsel gezielt zu modifizieren. In diesem Kontext sind Methyljasmonat (MeJA), Wasserstoffperoxid (H_2O_2) und Salicylsäure (SA), die Bestandteile unterschiedlicher Signaltransduktionsketten sind, sehr vielversprechende Kandidaten.

Das Ziel dieser Arbeit war es, den Einfluss von MeJA, H_2O_2 und SA auf das Spektrum der Monoterpen-Indolakaloide (MIAs) in *Vinca minor* zu erfassen. Dabei sollte vor allem analysiert werden, ob durch diese Behandlungen neue, bislang unbekannte Indolalkaloide gebildet werden. Diese Untersuchungen sollen klären, ob und in welchem Maße die Applikation von Wachstumsfaktoren und Signalsubstanzen eine aussichtsreiche Strategie sein kann, die Zusammensetzung pharmakologisch aktiver Naturstoffe zu verändern bzw. zu optimieren.

Zunächst musste eine zuverlässige Methode etabliert werden, um die stressinduzierten Veränderungen in der Zusammensatzung der MIAs einwandfrei bestimmen zu können. Dazu wurden zunächst die Alkaloide aus gesunden und ungestressten *V. minor* Blättern extrahiert und mittels HPLC fraktioniert. Auf der Basis von HRMS und NMR konnten alle für *V. minor* beschriebenen Hauptalkaloide identifiziert werden; dabei handelt es sich um Vincamin, Strictamin, 10-Hydroxycathofolin und Vincadifformin.

Die Behandlung der *V. minor* Pflanzen mit MeJA und H_2O_2 änderte das Spektrum der Indolalkaloide frappierend. Zwar blieben die Konzentrationen von 10-Hydroxycathofolin und Strictamin nahezu unverändert, doch die Gehalte von Vincamin und Vincadifformin

reduzierten sich drastisch. Des Weiteren resultierte aus der Applikation der Signalsubstanzen die Akkumulation drei anderer MIAs, die als Minovincinin, Minovincin, 9-Methoxyvincamin identifiziert werden konnten. Bei einem weiteren Alkaloid, dessen Stuktur noch nicht endgültig aufgeklärt werden konnte, handelt es sich wahrscheinlich um Kopsinin. Das Vorkommen von Minovincinin und Minovincin in *V. minor* war bekannt; allerdings wurde diesbezüglich lediglich das Vorhandensein von Spuren dieser Alkaloide berichtet. Beim 9-Methoxyvincamin handelt es sich um ein bislang noch nicht beschriebenes, und damit um ein neues Indolalkaloid.

Aufgrund des sehr großen strukturellen Ähnlichkeiten von Vincamin and 9-methoxyvincamin und deren gegenläufigen Veränderungen ihrer Konzentrationen, wurde postuliert, dass Vincamin in 9-Methoxyvincamin umgewandelt wird. In gleicher Weise wird vermutet, dass Vincadifformin zunächst zu Minovincinin und dann zu Minovincin modifiziert wird. Diese Umwandlungen erfordern oxidative Enzyme. In Analogie zu den bereits bekannten Enzymen, die für die Biosynthese der MIAs in *C. roseus* verantwortlich zeichen, sollte es sich auch bei diesen Enzymen um Cytochrom P450-Enzymen handeln. Um diese Vermutung zu verifizieren, wurden in weiteren Versuchsreihen - parallel zur Behandlung mit MeJA – zusätzlich spezifische Inhibitoren eingesetzt, von denen bekannt ist, die Alkaloid Biosynthese in *C. roseus* zu hemmen. Wie erwartet, ergab die gleichzeitige Gabe der Signaltransduktoren (MeJA und H_2O_2) und der Enzyminhibitoren (Resveratrol, Naproxen) an *V. minor*, dass sowohl die Umwandlung von Vincamin zu 9-Methoxyvincamin als auch die von Vincadifformin über Minovincinin zu Minovincin unterdrückt wurde. Damit bestätigen diese Ergebnisse eindrücklich, die stressinduzierte, oxidative Umwandlung der Hauptalakloide aus *V. minor*, nämlich Vincamin und Vincadifformin, zu 9-Methoxyvincamin, bzw. zu Minovincinin und Minovincin.

Diese stressinduzierten Umwandlungen und die Entdeckung von 9-Methoxyvincamin zeigen, dass selbst in intensive untersuchten Arzneipflanzen wie *V. minor*, noch immer eine vielversprechende Quelle für die Entdeckung neuer pharmakologisch aktiven Naturstoffe sein kann. Dementsprechend sollten zukünftig stressinduzierte Änderungen des Natursoff-Spektrums, vor allem solche, die durch die Applikation von MeJA oder anderen Wachtumsregulatoren oder Signaltransduktoren induziert werden, bei der Suche nach neueen bioaktiven Substanzen berücksichtigt werden. Diese neuen Herangehensweise sollte viel neue Möglichkeiten eröffnen, die Suche nach neunen pharmakologisch aktiven Naturstoffen zu erleichten und deren Erfolg zu steigern. Zusätzlich zur Applikation von Wachtumsregulatoren oder Signaltransduktoren bietet auch der Einsatz von Enzyminhibitoren eine weitere Möglichkeit, um den Gehalt und die

Zusammensetzung von Naturstoffen in Arzneipflanzen gezielt beeinflussen und verändern zu können.

Auch in Hinblick auf die Untersuchungen zu den Cytochrome P450-Enzymen, die für die stressinduzierte Umwandlung der MIAs verantwortlich sind, ist die Kombination von Enzyminhibitoren und Signaltransduktoren ein effektiver und vielversprechender Ansatz, der bei zukünftigen Forschungen zum Einsatz kommen sollte.

In dieser Arbeit wurde gezeigt, dass die Applikation von Wachtumsregulatoren und Signaltransduktoren den Gehalt und die Zusammensetzung von Naturstoffen drastisch verändern kann. In Kombination mit dem Ansatz einer "dereplication-guided" Isolierung der induzierten Substanzen, sollte ein solches Vorgehen zukünftig zur Entdeckung weiterer neuer Wirkstoffe, selbst in gut untersuchten Medizinalpflanzen, beitragen.

Abstract (Arabic)

الملخص العربى:

تحوير محتوى قلويدات الإندول فى نبات العناقية الصغرى

إن التغيير في محتوى وتكوين المنتجات الطبيعية يكون دائماً نتاجاً لعملية التوازن بين التخليق الحيوي، وإعادة توزيع وتكسير هذه المركبات. وتتأثر هذه العمليات الثلاثة بكلٍ من العوامل البيئية ومراحل النمو المختلفه للنباتات. ونتيجة لذلك ، قد يكون التأثير الكلي لهذه العوامل على محتوى الأيض الثانوي معقدًا ومتنوعًا. و بالتالي ، فإن هذه الاعتبارات تفتح أبوابا جديدة وطرق متعددة لتغيير وتعديل مركبات الأيض الثانوية ، وتفتح المجال للبحث عن مصادر جديدة للأدوية.

و بالنظر إلى تأثير العوامل البيئية المختلفة على النباتات، يبدو أن افتعال حالات الإجهاد المختلفة و الدراسة المتزامنة لمراحل النمو يشكلان نهجاً واعداً لتعديل مركبات الأيض الثانويه. ولهذا ، يجب أن يتم تعزيز و زيادة القدره الانتاجيه للنبات من هذه المركبات من خلال استخدام منظمات النمو المختلفه أو ناقلات الإشارات المشاركة في عملية التحكم فى استجابة النباتات للإجهاد. وفي هذا السياق ، يعتبر الميثيل جاسمونات (MeJA) و بيروكسيد الهيدروجين (H_2O_2) و حمض الساليسيليك (SA) أكثر المركبات أهمية فى سلسلة نقل الاشارات والتى ينتج عنها تعديل مركبات الأيض الثانوية.

الهدف من هذه الأطروحة هو تقييم تأثير MeJA وSA و H_2O_2 على تحوير محتوى وتكوين قلويدات الإندول فى نبات العناقية الصغرى (*Vinca minor*). وعلى وجه الخصوص ، دراسة قدرة هذه المعالجات على تكوين قلويدات إندول جديدة غير معروفة من هذا النبات. كما تهدف هذه الدراسه الى اثبات مدى امكانية تطبيق استخدام منظمات النمو المختلفة أو ناقلات الإشارات كإستراتيجية جديده لتحوير أو تحسين المحتوى الكيميائي للمنتجات الطبيعية.

و في هذا السياق و بهدف تحديد القلويدات الجديدة المحتملة التي يسببها الإجهاد ، أولاً ، تم تحديد الطيف الكروماتوجرافي للقلويدات (MIAs) من نباتات صحية وناضجة. وتم ذلك عن طريق استخلاص المحتوى القلويدي (MIAs) وتجزئته وفصله بواسطة الاستشراب السائلي عالي الأداء (HPLC). و تم التعرف على البنية التركيبية للقلويدات المفصولة باستخدام مطياف الكتلة عالى الدقه (HRMS) ومطياف الرنين النووي المغناطيسي (NMR)، و قد تم تحديد القلويدات الرئيسية مثل فينكامين (vincamine)، إستركتامين (strictamine)، 10-هيدروكسي كاثوفولين (10-hydroxycathafoline)، و فينكاديفورمين (vincadifformine).

و أوضحت الدراسة أن معالجة النباتات ب MeJA و H_2O_2 قد أدى إلي تغيير نمط وتكوين قلويدات الإندول الموجودة في نبات العناقية الصغرى. و على الرغم من أن تركيزات استركتامين (strictamine) و هيدروكسي كاثوفولين (10-hydroxycathafoline) لم تتغير تقريباً، فإن مستويات الفينكامين (vincamine) و فينكاديفورمين (vincadifformin) انخفضت بشكل كبير. وعلاوة على ذلك ، أدت معالجة النباتات ب (H_2O_2, MeJA) إلى تراكم أربعة من القلويدات (MIAs) الأخرى ، والتي تم تحديدها على أنها مينوفينسينين (minovincinine)، مينوفينسين (minovincine)، و 9-ميثوكسى فينكامين (9-methoxyvincamine). ويعتقد أن الرابع الذى لم يتم تحديد هيكله بشكل قاطع هو الكوبسينين (kopsinine). في حين أنه من المعروف بالفعل أن النبات قيد الدراسة يحتوى على مينوفينسين (minovincine) و مينوفينسينين (minovincinine)، ولكن بكميات ضئيلة جدا ،بينما يمثل 9-ميثوكسي فينكامين (9-methoxyvincamine) مركباً طبيعيًا جديدًا.

و استناداً إلى تشابه التركيب الكميائى للفينكامين (vincamine) و 9-ميثوكسي فينكامين (9-methoxyvincamine) و تغييراتهما العكسية في التركيزات، يفترض أن الفينكامين (vincamine) يتحول إلى 9-ميثوكسي فينكامين (methoxyvincamine). وبالمثل ، يعتقد أن فينكاديفورمين (vincadifformine) يتم تحويله أولاً إلى مينوفينسينين (minovincinine) وأخيراً إلى مينوفينسين (minovincine)، وتتطلب هذه التغييرات أنزيمات مؤكسدة. وبالنظر إلى الإنزيمات المقابلة المسئوله عن عملية التخليق الحيوي لقلويدات الإندول الموجودة فى نبات البفتة او الونكا (*Catharanthus roseus*) ، يبدو أنه من المرجح جدا أن تكون هذه التفاعلات محفزة بواسطة إنزيمات سيتوكروم بي 450. ومن أجل التحقق من هذا الافتراض، وبالتوازي مع معالجة النبات ب MeJA ، تم أيضاً معاملته بمثبطات إنزيمات معينة معروفة بتثبيطها أو تعديلها للتكوين الحيوي للقلويدات في نبات الونكا. وكما هو متوقع ، فإن معالجة النبات بريسفيراترول (resveratrol) أو نابروكسين (napoxen) ، على التوالي ، أدى الى منع تحويل الفينكامين (vincamine) إلى 9-ميثوكسي فينكامين (9-methoxyvincamine) وكذلك منع تحويل فينكاديفورمين (vincadifformine) عن طريق مينوفينسينين (minovincinine) إلى مينوفينسين (minovincine). وبناءً عليه، تؤكد هذه النتائج بوضوح أن الإجهاد يمكنه أن يحث التحويل التأكسدي لكل من قلويدات الإندول الرئيسية الموجودة فى نبات العناقية الصغرى.

و مما سبق نستطيع أن نخلص إلى أن التحولات الكميائيه الناتجه عن الإجهاد، وخاصة اكتشاف 9-ميثوكسي فينكامين (-9) methoxyvincamine كمنتج طبيعي جديد، يظهر بوضوح أن النباتات المعروفه التى تم دراستها على نطاق واسع مثل نبات العناقية الصغرى، قد يكون مصدرًا لاكتشاف عقاقير كيميائية جديدة. وبناءً على ذلك، فانه يجب في المستقبل أثناء البحث عن مركبات نشطة بيولوجيًا أن يتم دمج الإجهاد الذى ينتج عنه تحويرات في تكوين المنتجات الطبيعية، ويفضل أن يتم ذلك بمعاملة النباتات ب MeJA وغيره من منظمات النمو. ومثل هذا النهج قد يفتح أبوابًا جديدة لتحسين و اكتشاف مركبات دوائية جديدة. و بالإضافة إلى تطبيق منظمات النمو أو ناقلات الإشارة، كما يمكن أيضاً استخدام مثبطات الإنزيمات كنهج بديل لتحوير المحتوى الكيميائى للمركبات النشطة بيولوجياً في النباتات.علاوة على ذلك، يمثل استخدام المعالجات التي تجمع بين مثبطات إنزيمات السيتوكروم بى 450 المسؤولة عن تحوير قلويدات الإندول ومنظمات النمو أو ناقلات الإشارات أثناء عملية الاجهاد أداةً فعالة ينبغي استخدامها في الأبحاث المستقبلية.

وفقا لذلك ، تبين أن تطبيق منظمات النمو وناقلات الإشارات أثناء عملية الاجهاد يمكن أن يغير بشكل جذري محتوى الأيض الثانوي للنبات و الذي قد يسهم فى تكوين منتجات طبيعية جديدة. بالاضافة لذلك فإن فصل المركبات الناتجة عن الاجهاد باستخدام نظام "dereplication-guided isolation" وبالتالى يمكن لهذا النهج أن يعزز اكتشاف عقاقير جديدة، حتى في النباتات الطبية التي التى تم دراستها على نطاق واسع.

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Abbreviation

ABA	Abscisic acid
ACN	Acetonitrile
AOC	Allene oxide cyclase
AOS	13-Allene oxide synthase
AS	Anthranilate synthase
brs	broad singlet
$CHCl_3$	Chloroform
COSY	COrrelation Spectroscopy
CYP	Cytochrome P450
d	doublet
Da	Dalton
DMAPP	Dimethylallylpyrophospate
DMSO	Dimethylsulfoxide
DW	Dry weight
DXR	1-deoxy-D-xylulose-5-phosphate reducto-isomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
ESI	Electrospray ionization
ET	Ethylene
Et_3N	Triethylamine
g	gram
G10H	Geraniol 10-hydroxylase
GES	Geraniol synthase
GPP	Geranyl diphosphate
GPPS	Geranyl diphosphate synthase
h	hour
H_2O_2	Hydrogen peroxide
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High-performance liquid chromatography
HR	High-Resolution

HSQC	Heteronuclear Single Quantum Coherence
Hz	Hertz
IDI	Isopentenyl diphosphate isomerase
IPAP	Internal phloem associated parenchyma
IPP	Isopentenyl pyrophosphate
IS	Internal standard
ISR	Induced systemic resistance
J	Coupling constant
JA	Jasmonic acid
JAs	Jasmonates
LAMT	Loganic acid *O*-methyltransferase
LC	Liquid chromatography
LOX	13-Lipoxygenase
LSD	Least significant difference
M	Molar
m	multiplet
m/z	Mass-to-Charge ratio
MeJA	Methyl jasmonate
MeOH	methanol
MEP	2-*C*-methyl- D-erythritol-4-phosphate or Non-mevalonate pathway
mg	milligram
MIAs	Monoterpene indole alkaloids
min	minute
mL	milliliter
MS	Mass spectroscopy
MS/MS or MS^2	Tandem mass spectrometry
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NH_4OAc	Ammonium acetate
NMR	Nuclear magnetic resonance
NO	Nitric oxide

NOESY	Nuclear Overhauser Effect SpectroscopY
OPDA	12-Oxo-phytodienoic acid
OPR	12-Oxo-phytodienoic acid reductase
PDA	Photodiode array
PDE-1	Phosphodiesterase-1
pH	potential of hydrogen
ppm	parts per million
PR1	Pathogenesis-related 1
q	quartet
RDA	Retro Diels Alder
rel. int.	Relative intensity
ROESY	Rotating frame Overhause Effect SpectroscopY
ROS	Reactive oxygen species
RP	Reversed phase
rpm	Revolution per minute
RT or t_R	Retention time
s	second
s	singlet
SA	Salicylic acid
SAR	Systemic acquired resistance
SGD	Strictosidine glucosidase
SLS	Secologanin synthase
SM	Secondary metabolite
STR	Strictosidine synthase
T	temperature
t	triplet
TDC	Tryptophan decarboxylase
TEA	Triethylamine
TFA	Trifluoroacetic acid
TOCSY	TOtal Correlated SpectroscopY
UHPLC-MS	Ultra-high-pressure liquid chromatography-mass spectrometry

UHR-TOF-MS	Ultra-high-resolution time-of-flight mass spectrometry
UV	Ultraviolet
λ	Wavelength
δ	Chemical shift in ppm
$[\alpha]^{T}_{D}$	Optical rotation at **λ** = 589 nm and temperature T
°C	Degree Celsius
µL	microliter

Chapter 1: Introduction

Plants are sessile; they have no capacity to move from an adverse environment or to escape from an herbivore. Their only chance for survival is defense. This could be accomplished either mechanically, e.g. by creating barriers, thorns, and spines, or chemically. Chemical defense is based on the production of a tremendous array of different pungent, acrid or toxic substances. The biosynthesis of these compounds had been evolved during the entire plant phylogeny. In this context, the ecological interaction of plants with herbivores, pathogens, and unfavorable abiotic factors had been the driving force to select for both, general and specific defense compounds (Pichersky and Gang, 2000; Fini *et al.*, 2017). The related protective effects, frequently simplifying denoted as defense function, entail selection advantages which finally lead to the establishing in the population. Many of these compounds reveal strong effects also on human metabolism, and accordingly, they are used for medicinal purposes. However, we always have to be aware: "*Plants do not produce drugs*" (Littleton, 2007). The production of the related natural compounds always has to be evaluated with respect to their ecological significance. Nevertheless, the related effects make them very useful as drugs. Due to the tremendous number of different compounds, up to now, the pharmacological effects of only a limited number of substances are investigated (Atanasov *et al.*, 2015).

1.1 Plant defense – an intriguing source for drug discovery

As outlined, it is well known that plants generate a large number of new, natural products, which also might reveal high therapeutic value (Pan *et al.*, 2013; Atanasov *et al.*, 2015). However, the lack of reproducibility reported for more than 40% of related plant extracts is considered as one of the major drawbacks in using plants in pharmaceutical drug discovery (Cordell, 2000). Moreover, the biochemical profiles of plants harvested at different times and locations vary greatly (Poulev *et al.*, 2003; Atanasov *et al.*, 2015). With respect to defensive compounds, such variability is not surprising; in principle, there are two different mechanisms related to the production and accumulation of defense compounds. First, the so-called preexisting defense compounds also called phytoanticipins. These compounds constitutively are present in the plants, either already as active compounds like alkaloids, saponins or various phenolic compounds, or they are activated postmortem from pre-existing precursors like cyanogenic glucosides or glucosinolates (Matile, 1980;Tomas-Barberan and Gil, 2008; Iriti and Faoro, 2009). Secondly, the active compounds are produced only when the plant is stressed by biotic or abiotic factors. In this manner, for example, stilbenes are produced in response to high irradiation (Marti *et al.*, 2014), or phytoalexins are synthesized *de novo* when the plant is

attacked by fungi or bacteria (Pusztahelyi *et al.*, 2015; Jeandet, 2015). Notwithstanding, both mechanisms are not categorically separated, i.e., the contents of phytoanticipins could also be enhanced in response to stress. In this sense, the concentration of nicotine in leaves *Nicotiana attenuata* attacked by the larvae of *Manduca sexta* strongly is enhanced due to a stress-related induction of alkaloid biosynthesis (Winz and Baldwin, 2001; Steppuhn *et al.*, 2004). Furthermore, the concentration of various natural products accumulated in numerous spice and medicinal plants is enhanced in response to drought stress (for review see (Kleinwächter and Selmar, 2014). In addition to these quantitative changes, stress may also alter the composition of the relevant compounds (Poulev *et al.*, 2003; Wojakowska *et al.*, 2013; Sampaio *et al.*, 2016), like it was demonstrated for changes of phenolic profiles in the reaction of lupin (*Lupinus angustifolius*) plants to infections with the pathogenic fungi *Colletotrichum lupini* (Wojakowska *et al.*, 2013) . The composition of the volatile oil in poplar cuttings (*Populus* × *euramericana* 'Nanlin 895') was altered in response to methyl salicylate (Tang *et al.*, 2015). In the same sense, the application of methyl jasmonate (MeJA) leads to a significant increase in the content of indole alkaloids in hairy root cultures of *Catharanthus roseus*, and to a shift in their composition (Rijhwani and Shanks, 1998; Rodriguez *et al.*, 2003). Accordingly, the quantity, as well as the composition of healthy and non-stressed plants, could be quite different than those of the plants elicited by various biological and abiotic stress situations. Up to now, these coherences have not been taken into account adequately, when searching for novel pharmacological active natural products. Consequently, approaches for plant drug discovery may have been – at least in part - biased so far. We have to consider that a sound search for bioactivity requires that the wild plant species of interest should not only be analyzed exclusively on the basis of healthy adult individuals, but also the analyses of stressed individuals have to be implemented.

A further aspect, which has to be considered, when comprehensively is searched for novel pharmacologically active compounds, is related to the plant development. The content, as well as the composition of natural compounds strongly depends on phenological stages, maturation and senescence processes (Skursky *et al.*, 1969; Brown *et al.*, 2003; Nezhadali *et al.*, 2014). A very popular example for massive variations in the content of relevant compounds due to developmental differences is visualized by the well-known phenomenon of spice plants: the favour and thus the quality of the related material is much higher, when young leaves are used than in the case of mature ones, since the concentration of the aromatic monoterpene is far higher in young than in old leaves.

1.2 Objectives and approaches

Based on the insights mentioned above, we can conclude: a thorough and comprehensive search for novel pharmacologically active compounds should include the application of various stress situations and the consideration of different developmental stages (Figure 1-1).

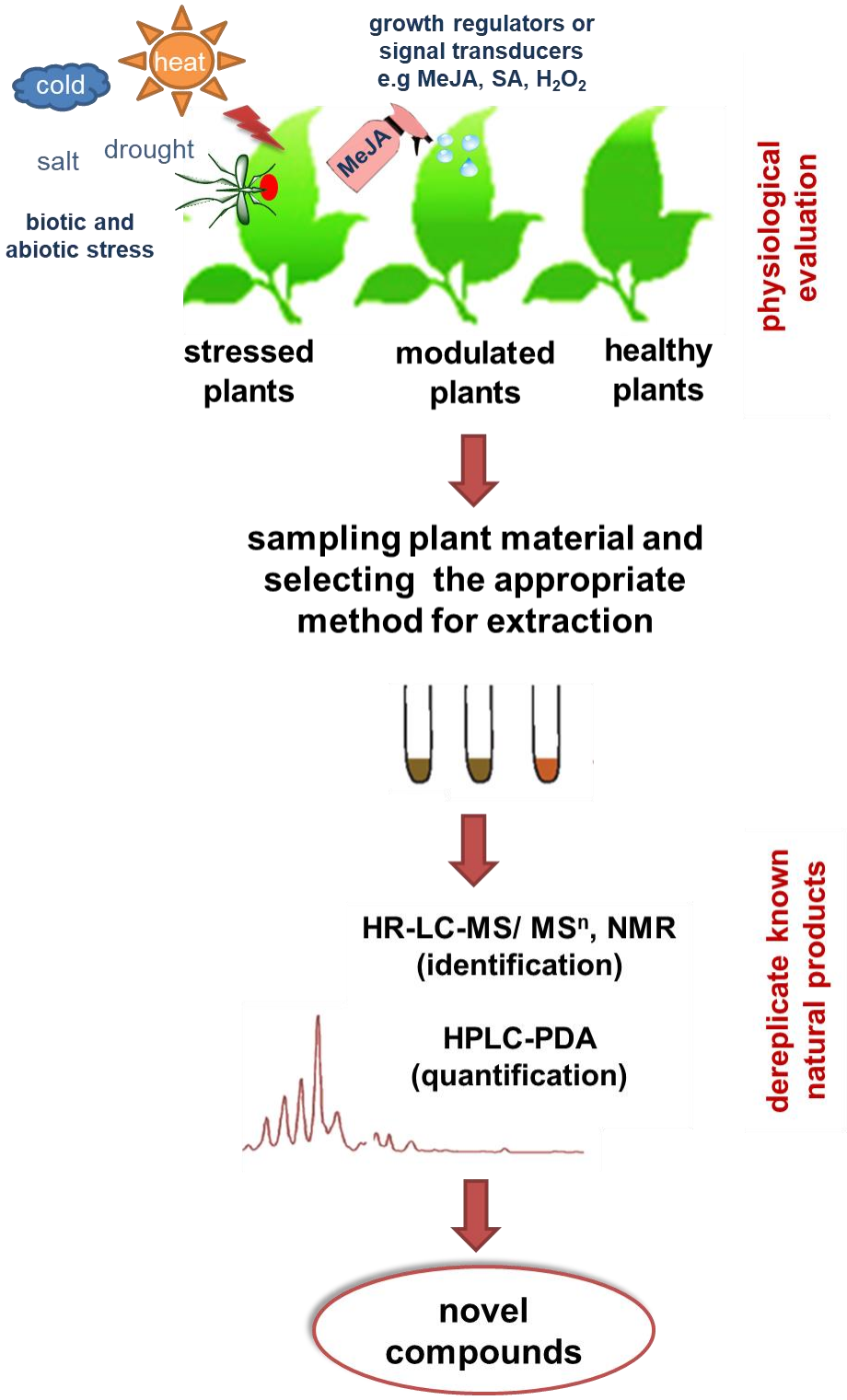


Figure 1- 1: Schematic overview of the novel approach to study the effects of stress and growth regulators as promising tools for phytochemical drug discovery.

An alternative approach covering these requirements is to mimic the stress and the developmental processes by the use of growth regulators or inducers (Figure 1-1). In this sense, MeJA, hydrogen peroxide (H_2O_2), and salicylic acid (SA) have been used to induce characteristic stress responses, and also impacts secondary metabolism. An intriguing example of such approach is based on the analyses of the indole alkaloids of *C. roseus* treated with MeJA. When seedlings of *C. roseus* are treated with this signaling substance, the concentration of indole alkaloids is changed significantly and its composition is shifted (Aerts *et al.*, 1994), for further details see the Scientific background. Another member of the Apocynaceae family, representing also an important medicinal plant that contains pharmacological important indole alkaloids, is lesser periwinkle (*Vinca minor*). Up to now, no information on putative stress-related changes of the *V. minor* alkaloids is available. Accordingly, MeJA treatment on *Vinca* plants seems to be an appropriate and promising approach for identifying novel pharmacological products.

In this sense, the major goal of this thesis is the elucidation of the impact of MeJA on the alkaloid metabolism in *V. minor*. The project is aimed to comprehend the effect of MeJA on the pattern of indole alkaloids of *V. minor* with a special emphasis on the appearance of novel, so far unknown indole alkaloids. In this manner, a new approach to identify pharmacologically active compounds in *V. minor* is introduced and thus, a novel strategy in plant phytochemical drug discovery is presented.

To achieve these goals, different lines of experimental approaches have been followed:

1- Phytochemical analyses of healthy, non-stressed *V. minor* plants, i.e., identification, isolation and structural elucidation of the major indole alkaloids.
2- The phytochemical analyses (identification, isolation and structural elucidation) of the major alkaloids of *V. minor* plants, when treated with different plant growth regulators, such as MeJA, salicylic acid, and hydrogen peroxide.
3- Establishing a reliable method for quantification of the relevant indole alkaloid(s) in *V. minor*.
4- Studying the effect of inhibitors (e.g. naproxen and resveratrol) on the MeJA-induced conversion of indole alkaloids

1.3 Thesis outline

The current thesis is divided into six chapters. Apart from the current introduction (**chapter 1**), the other four sections are:

Chapter 2: The **Scientific background** presents a comprehensive review and the current knowledge about chemistry, biological activity, biosynthesis and regulation of indole alkaloids of *V. minor* plants. In addition, this literature survey focuses on the biosynthetic and regulation processes of indole alkaloids in well-studied *C. roseus* plant in order to create the basis for the designed experimental approaches with *V. minor*.

Chapter 3: The **materials and methods** display the experimental procedures which were used for evaluating the impacts of application of growth regulators and signal transducers (e.g. MeJA, SA, and H_2O_2) on the spectrum and composition of indole alkaloids in *V. minor* leaves.

Chapter 4: The **Results** present the outcome of this study, which vividly demonstrate that artificial elicitation indeed leads to massive qualitative as well as quantitative variations in the content of bioactive compounds. In consequence, the results verify that the utilization of growth regulators, especially of MeJA, could be a promising tool for altering and diversifying the composition of active substances in medicinal plants.

Chapter 5: in the **Discussion**, the different aspects of the various novel insights are contemplated and evaluated in detail. In addition, suggestions for further directions in phytochemical drug discovery are outlined.

Chapter 6: in the **Conclusion** based on the results, novel insights, deductions, and promising strategies for both, the improved search for novel pharmacological agents and the elucidation of biosynthetic pathways will be presented.

Chapter 2: Scientific background

The intention of this study was to investigate whether or not the application of growth regulators could be a novel strategy to modify the composition of active components in medicinal plants. In this study, mature *Vinca minor* (Apocynaceae) plants of defined physiological status were used as a model system to investigate the effect of elicitation with methyl jasmonate (MeJA) and other growth regulators. In this context, the applicability of using growth regulators or signal transducers as a promising tool for altering and diversifying the composition of active substances in *V. minor* was studied and evaluated. In the following chapter, the current scientific literature on the various aspects about chemistry, biological activity, biosynthesis and regulation of indole alkaloids of *V. minor* plant is reviewed. The major alkaloid in the leaves was reported to be vincamine, which has pronounced cerebrovasodilatory and neuroprotective activities (Vas and Gulyás, 2005; Hasa *et al.*, 2013). Despite the economic importance of *V. minor* alkaloids, little is known about their biosynthesis. However, its high genetic similarity to its close relative *Catharanthus roseus*, a model plant whose indole alkaloids biosynthesis pathways have been extensively studied (Thamm *et al.*, 2016; Pan *et al.*, 2016; Dugé de Bernonville *et al.*, 2015; O'Connor and Maresh 2006), can be exploited to identify the corresponding processes in *V. minor*. Accordingly, this literature survey focuses on the biosynthetic processes in *C. roseus* in order to create the basis for the designed experimental approaches with *V. minor*.

2.1 Classification of genus *Vinca*

The genus *Vinca* was originally established by Carl von Linné in 1735 (Farnsworth 1961). *Vinca* species were originally native to Europe but extended to North-Africa and the Near-East. Outside this area, there is only one species, i.e., *Vinca erecta*, which occurs in the East of Central Asia. Genus *Vinca* was first classified by Pichon followed by Pobedimova (Aynilian *et al.*, 1974). Later on, Stearn (Stearn, 1973) intensified this classification and mentioned 16 species belonging to 6 sections as follows:

Family: Apocynaceae
Subfamily: Plumieroideae
Genus: *Vinca* L.
Section: Herbacea
V. herbacea Waldst. et Kit.
V. pumila E. D. Clarke
V. libanotica Zucc.
V. sessilifolia DC.
V. bottae Jaub. & Spach.

V. mixta Vel.
V. hausknechtii Bornm. et Sint.
Section: Erecta
V. erecta Regel. et Schmalh.
Section: Major
V. major L.
subspecies: *major*
subspecies: *hirsuta* (Boiss.) Stearn
(syn. *V. pubescens* Urv.)
Section: Difformis
V. difformis Pourr.
subspecies: *difformis*
subspecies: *sardoa* Stearn
Section: Minor
V. minor L.
Section: Balancia
V. balancia Penzes.

According to Aynilian et al. (1974), the taxonomy of the genus *Vinca* was not fully coherent. Based on the great variety of indole alkaloids present in the various *Vinca* species, several previous chemotaxonomic attempts had been made. The first of these studies was conducted by (Paris and Moyse, 1957) followed by Janot and co-workers (1965) pointed out that *V. minor* contains mainly alkaloids of class B (Aspidospermine type), while other *Vinca* species contained alkaloids of class B and class A (Yohimbine type), the latter prevailing regularly. Alkaloids of class C (Iboga type) and dimeric bases of the mixed B-C classes, were absent in *Vinca* and seemed to be characteristic of the genus *Catharanthus* by studying the distribution of indole alkaloids in *Vinca* species (Janot *et al.*, 1965; Figure 2-1).

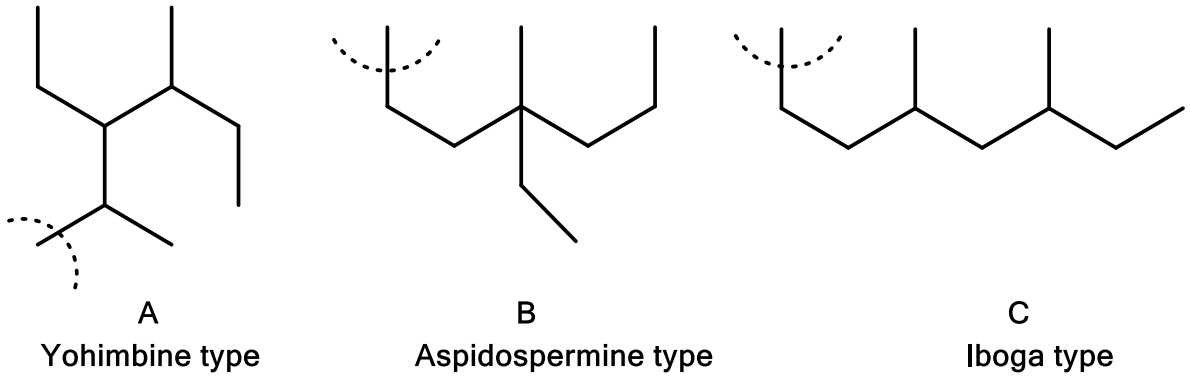


Figure 2- 1: Characteristic types of indole alkaloids present in the genus *Vinca.*

A comprehensive study on the occurrence of the different structural type of indole alkaloid was published by Novacek & Stary (1973) see Table 2-1.

Table 2- 1: Classification and distribution of A and B classes of indole alkaloids in the genus *Vinca.*

Section	Species	Number of characterized alkaloids	Class A	Class B	Predominant class
1. Minor	1. *V. minor* L.	39	7	32	B
2. Major	2a. *V. major* L.	12	9	3	A
	2b. *V. difformis* Pourr.	9	6	3	A
	2c. *V. pubescens* Urv.	3	3	0	A
3. Herbacea	3a. *V. herbacea* Waidst. et Kit.	19	15	4	A
	3b. *V. libanotica* Zucc.	12	9	3	A
	3c. *V. haussknechtii* Bornm. et Sint.	-	-	-	-
4. Erecta	4. *V. erecta* Regel. et Schmalh.	44	24	20	A

Based on the results of their chemotaxonomic studies and on a critical evaluation of published data on genus *Vinca* systematic, Trojanek et al. (1973) divided the genus *Vinca* into 4 sections, comprising 8 species (Table 2-1). This classification agrees well, not only with the distribution of the basic alkaloid classes A and B but also with that of corresponding structural subgroups within these classes. A careful examination of Table 2-1 shows that there is justification for devoting a separate section to *V. minor* since it is the only species able to synthesize about 80% of its alkaloids as the B type.

2.2 Characterization of *Vinca minor* L.

Vinca minor is commonly known as myrtle or lesser periwinkle. It is a member of family Apocynaceae and considered as an evergreen perennial herbaceous plant (Robinson, 1870; Knight, 2007; Booy *et al.*, 2015). *V. minor* is a small subshrub (chamaephyte), non-native archaeophyte, which has been planted for centuries as an ornamental, medicinal and ritual plant (Čepková *et al.*, 2016). Although it represents a European species, *V. minor* has been cultivated in the Mediterranean region at least since Roman times. Yet, the exact natural range of its distribution is unknown (Meusel *et al.*, 1978). Today, the species is widely naturalised also in other continents. In Northern America, it is even regarded as an extremely problematic invasive species (Darcy and Burkart, 2002).

In general, *V. minor* represents a typical ground-cover plant with a high degree of stem rooting. Its maximal high is about 15 cm. In Central Europe, it only rarely produces fruits, and accordingly, *V. minor* predominantly is reproduced clonally by long stolons (Čepková *et al.*, 2016). Its evergreen leaves reveal a lanceolate to an elliptical shape and are arranged oppositely (Figure 2-2). They are pointed or blunt with a leathery texture and an entire margin. The young

leaves are yellowish green, whereas the mature ones are glossy dark green (Figure 2-2). The petioles are about 3-4 mm long. The flowers are solitary in the leaf axils. Due to an induction by short day conditions, flowers produced mainly from spring to early summer, however in mild falls there will also be few flowers produced. The flowers are about 9-11 mm long and 25-30 mm in diameter revealing a five-lobed corolla. Generally, they are violet-purple, however, in some cultivated selections, they also might be pale purple or white. The fruit is a pair of follicles, containing numerous seeds (Robinson, 1870; Miller, 2003).

The closely related species *Vinca major* is quite similar, but larger in size of all parts, with broader leaves and a hairy margin (Booy *et al.*, 2015).

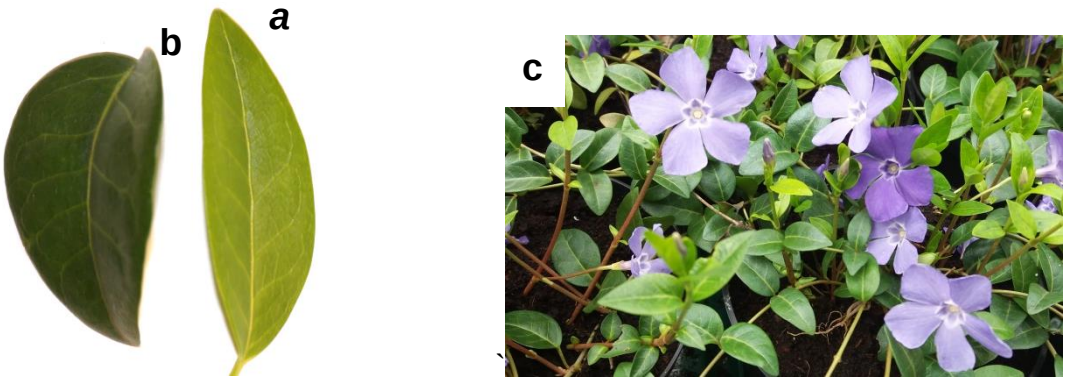


Figure 2- 2: *Vinca minor* L. - (a) old leaf; (b) young leaf; (c) habitus.

With respect to climatic adaptation, *V. minor* reveals some xeromorphic characters: the leaves of *V. minor* are thick and the outer cell wall of the upper epidermis is strongly cutinized with thread-like wax formations. The leaves reveal a thick, two-layered palisade chlorenchyma and fiber bundles in the veins. The non-articulated laticifers are unbranched and found only on the lower side of the vascular strands. They often contain spherical particles (Fjell, 1983). According to Chen et al. (2017), *V. minor* is resistant to cold, and its optimal growth environment temperature is 10 ^{0}C. In addition, the authors reported that *V. minor* might be better than *C. roseus* in the production of secondary metabolites based on the growth environment, leaf morphology characteristics, energy input and MIAs yields.

2.3 Indole alkaloids from *V. minor*

2.3.1 Structural variation of the *Vinca* alkaloids

The alkaloids of *Vinca minor* had been studied thoroughly by several research groups in Europe. Up to now, more than 50 indole alkaloids have been extracted and isolated from lesser periwinkle (Taylor, 1965; Aynilian *et al.*, 1974; Proksa and Grossmann, 1991). Although

different studies frequently reveal quite different compositions of indole alkaloids of *V. minor*, in all cases, the major alkaloid in the leaves was reported to be vincamine (eburnamine-type, 25-65%) (Proksa and Grossmann, 1991; D'Amelio Sr. *et al.*, 2012; Hasa *et al.*, 2013). Apart from vincamine, many different groups are outlined. The three main groups: eburnamine (e.g. vincamine), aspidospermine (e.g. vincadifformine) and akuammiline (e.g. strictamine) groups are displayed in Figure 2-3. The detailed presentation of all 62 different structures is given in (Table A-1) in the appendix. Kaczmarek and Lutomski, (1965) reported that *V. minor* collected between the middle of April and June contained the most of alkaloids with hypotensive activity, and is, therefore, suitable for pharmaceutical purposes.

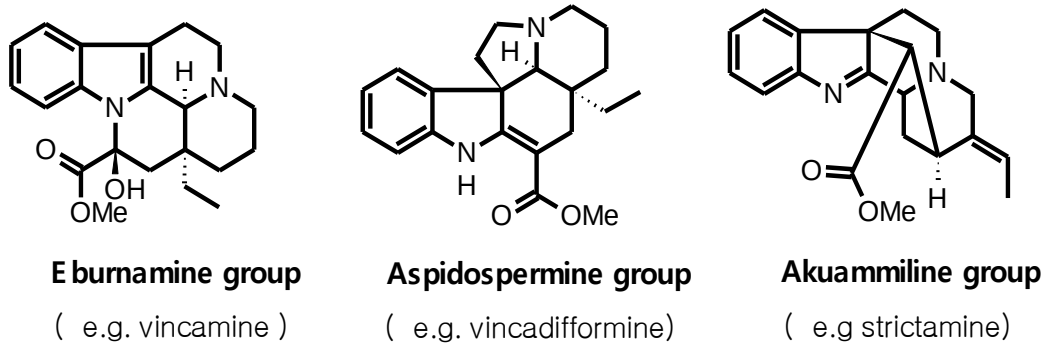


Figure 2- 3: The major groups of indole alkaloids in *Vinca minor.*

2.3.2 Biosynthesis of indole alkaloids – general aspects

As the result of numerous very early feeding experiments, it turned out that the C-9 and C-10 moiety of the monoterpene indole alkaloids (MIAs) were derived from the acetate-mevalonate pathway to yield the iridoid skeleton, which was incorporated into indole alkaloids whose skeleton derived from tryptamine. It was postulated that the aliphatic C-9 and C-10 units are incorporated in three different basic forms to produce the three basic classes of indole alkaloids mentioned in Figure 2-1 (Aynilian *et al.*, 1974). Meanwhile, using *C. roseus* as model plant, the biosynthesis of MIA is comprehensively elucidated (Thamm *et al.*, 2016; Pan *et al.*, 2016; Dugé de Bernonville *et al.*, 2015; O'Connor and Maresh 2006). In contrast, despite its high economic importance, the corresponding biosynthesis of the alkaloids from *V. minor* is poorly studied. Nonetheless, due to the high genetic similarity between these both plants, the ample knowledge on the alkaloid biosynthesis in *C. roseus* can be exploited to identify the corresponding processes in *V. minor*. Accordingly, this literature survey focuses on the biosynthetic processes in *C. roseus* in order to create the basis for the designed experimental approaches with *V. minor*.

As proposed several decades ago, indole alkaloids are derived from the convergence of two primary metabolic routes: the shikimate and the secoiridoid pathways generating the indole and the terpene moiety of the universal MIA precursor, strictosidine (van der Heijden *et al.*, 2004; Verma *et al.*, 2012b). From strictosidine, a tremendous variety of different MIAs is derived (see Figure 2-4).

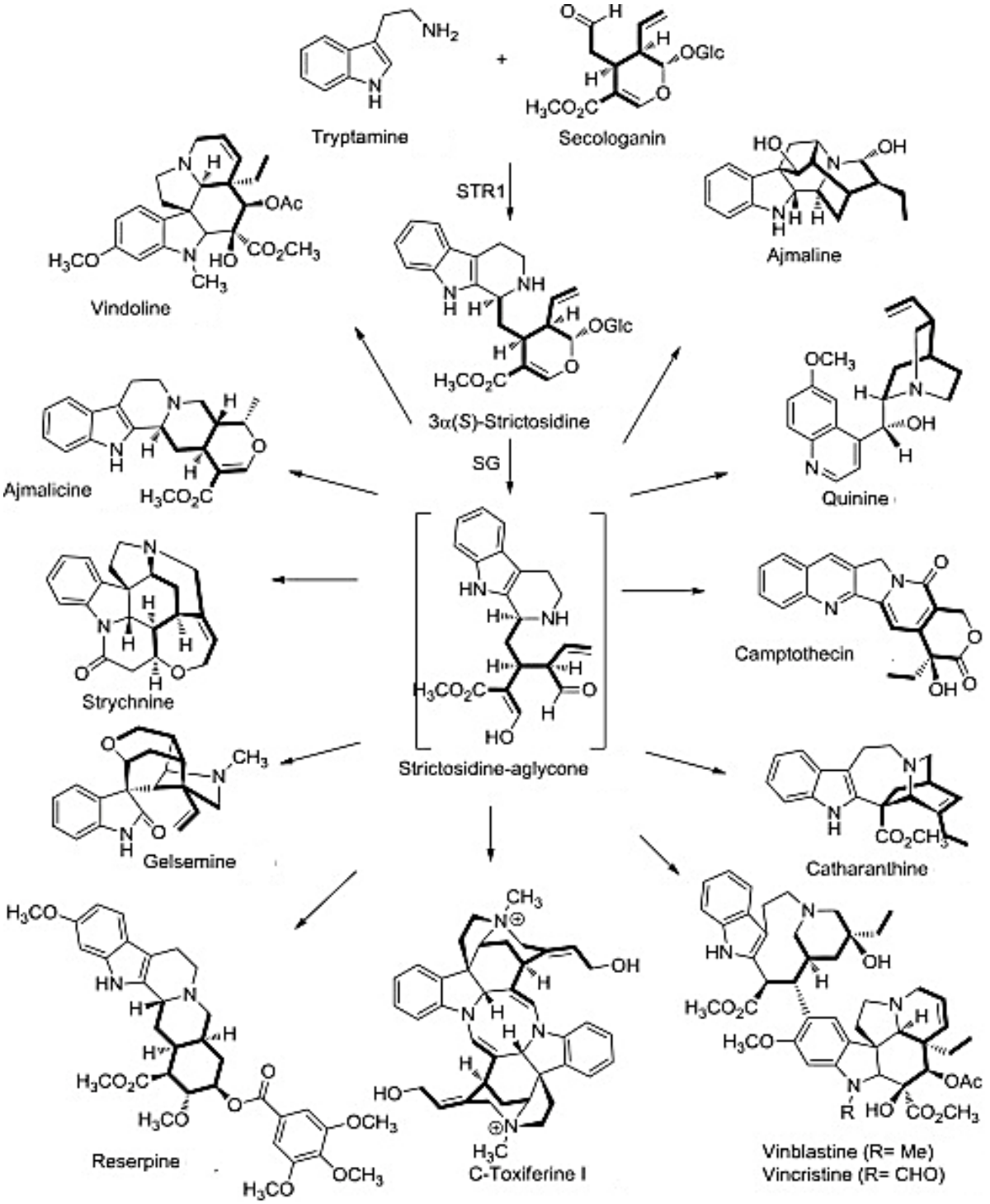


Figure 2- 4: Strictosidine as precursors for the great variety of indole alkaloids. The secologanin skeleton in bold highlights changes of the chemical structure arising by skeleton rearrangements during biosynthesis. STR1: strictosidine synthase; SG: strictosidine glucosidase (Wu *et al.*, 2016).

In principle, the biosynthesis of MIAs can be conveniently divided into four stages (Thamm *et al.*, 2016; Pan *et al.*, 2016):

a. The production of tryptamine

Tryptophan is produced by the shikimate–pathway, which subsequently is converted to yield tryptamine by the action of the tryptophan decarboxylase (TDC).

b. The production of secologanin (MEP and secoiridoid pathway)

- Isopentenyl pyrophosphate (IPP) and Dimethylallylpyrophospate (DMAPP) is predominantly[1] produced by the MEP pathway (2-C-methyl-D-erythritol-4-phosphate).
- The iridoid pathway that converts geraniol to secologanin.

c. The condensation of tryptamine and secologanin

The condensation of both educts is catalyzed by strictosidine synthase. The product, strictosidine, corresponds to the central precursor for most of the MIAs. Deglycosylation of strictosidine gives rise to a series of unstable intermediates for the biosynthesis of many different classes of MIAs.

d. Final modifications:

The unstable intermediates generated by the strictosidine deglucosylation are modified and converted to the corresponding final products.

2.3.2.1 The production of tryptamine

The shikimate pathway, a major biosynthetic route for both primary and secondary metabolism, is responsible for the generation of the aromatic amino acids. In principle, erythrose-4-phosphate and two molecules phosphoenolpyruvate combined to yield chorismate. Chorismate represents an important branching point leading either to phenylalanine and tyrosine or, via anthranilate, to tryptophan, respectively. According to Herrmann and Weaver, (1999), the key enzyme in tryptophan biosynthesis is the anthranilate synthase (AS). Yet, the enzymes converting anthranilate to tryptophan has not been studied in *C. roseus* so far. Further on, tryptophan is converted by tryptophan decarboxylase (TDC) to tryptamine which provides the indole moiety to MIAs (Figure 2-5; 2-6).

In the aerial tissues, the epidermis of the leaf has the maximum expression of the *TDC* gene. In the underground tissues, *TDC* transcripts are localized in protoderm and cortical cells around

[1] With respect to some former works, IPP and DMAPP used for the biosynthesis of secologanin could also be provided by the acetate-mevalonate pathways (Coscia *et al.* 1969)

the root apical meristem. The early steps of the tryptophan pathway are thought to occur in plastids. The TDC enzyme essentially operates in the cytosol. This implies that tryptophan has to move out from plastids to the cytosol for its decarboxylation by TDC to yield tryptamine, which is immediately transported to the cell vacuole for its subsequent condensation with secologanin (Figure 2-5; 2-6; (Zhu *et al.*, 2015; Pan *et al.*, 2016)).

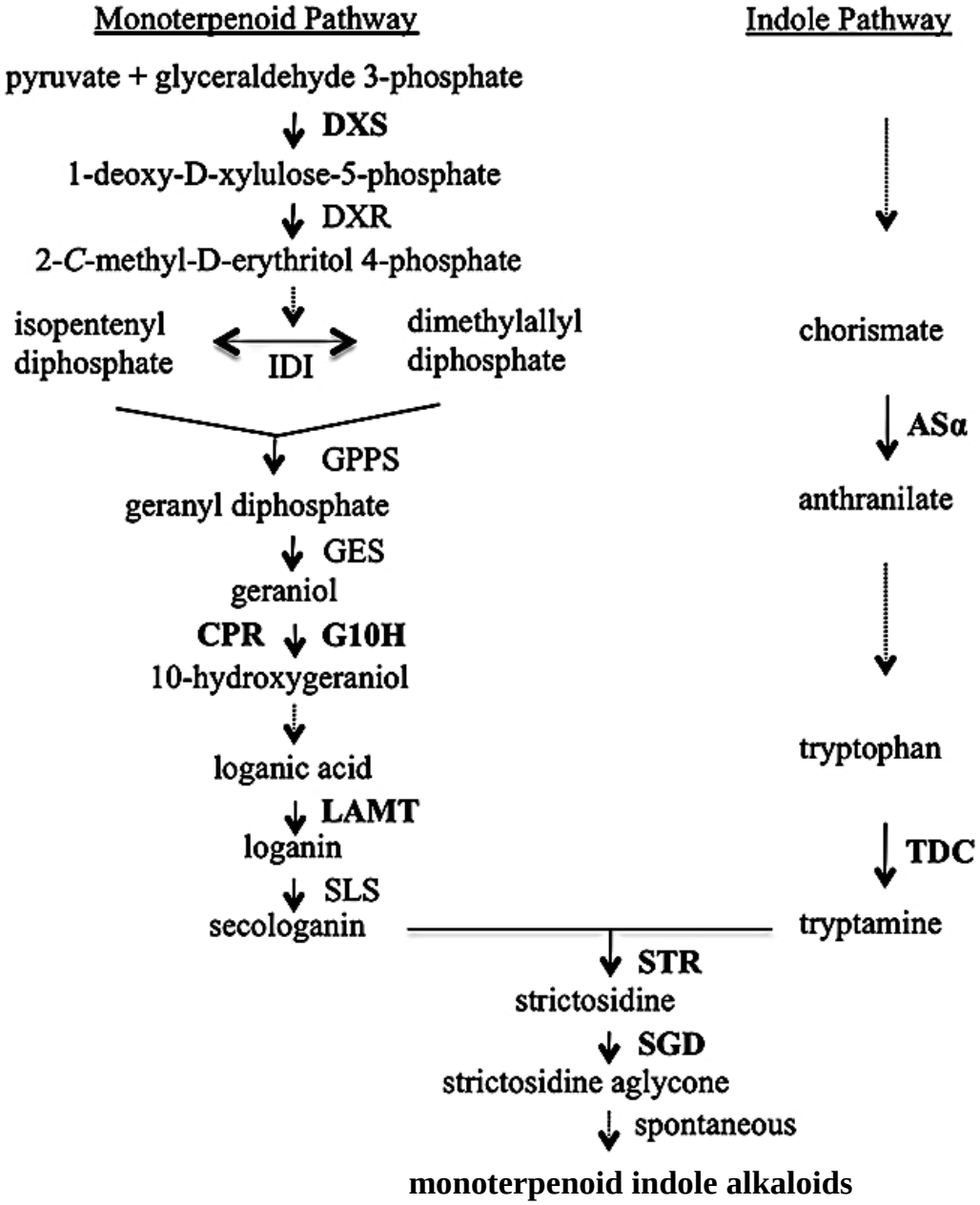


Figure 2- 5: Biosynthesis of *Catharanthus* monoterpenoid indole alkaloid. Enzymes are indicated using capital letters and metabolites using lowercase letters. Solid arrows indicate enzymatic conversions. AS: anthranilate synthase; DXR: 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS: 1-deoxy-D-xylulose-5-phosphate synthase; G10H: geraniol 10-hydroxylase; GES: geraniol synthase; GPPS: geranyl diphosphate synthase; IDI: isopentenyl diphosphate isomerase; LAMT: loganic acid *O*-methyltransferase; SGD: strictosidine glucosidase; SLS, secologanin synthase; STR: strictosidine synthase; TDC: tryptophan decarboxylase (Zhu *et al.*, 2015; Li *et al.*, 2015).

2.3.2.2 The production of secologanin

Production of activated isoprenes

Isopentenyl diphosphate[2] (IPP) and dimethylallydiphosphate[2] (DMAPP), the precursor for all terpenoids, are produced by either the mevalonate biosynthetic pathway (Coscia *et al.*, 1969) or the MEP (methylerythritol phosphate) pathway. The MEP pathway involves seven enzymatic steps, starting from the condensation of pyruvate with D-glyceraldehyde-3-phosphate ending with the production of IPP (El-Sayed and Verpoorte, 2007). The final product IPP is converted to dimethylallyl diphosphate (DMAPP) by isopentenyl diphosphate isomerase (IDI). The generation of geranyl diphosphate (GPP) results from the condensation of IPP and DMAPP by GPP synthase (GPPS) (Figure 2-5). ^{13}C labeling experiments followed by NMR proved that the mevalonate-independent pathway (MEP) is the major route for the biosynthesis of, secologanin in *C. roseus* (Contin *et al.*, 1998).

The primary locations for the expression of genes in the MEP pathway are the internal phloem associated parenchyma cells (IPAP) present in the periphery of stem pith or intraxylary on the upper part of the vascular bundles in leaves (Simkin *et al.*, 2013).

Secoiridoid pathway

In the first step of iridoid terpene biosynthesis, geraniol is hydroxylated by geraniol-10-hydroxylase (G10H). 10-hydroxygeraniol converted and glucosylated to yield loganic acid, which by the action of loganic acid methyltransferase (LAMT) is methylated to loganin. Finally, secologanin synthase (SLS) catalyzes the conversion of loganin to secologanin (Figure 2-5; 2-6).

The MEP pathway and early steps of the secoiridoid pathways take place in internal phloem associated parenchyma (IPAP) cells while the last two steps of the secoiridoid pathway are localized in the epidermis. Loganic acid is the mobile intermediate transferred from IPAPs to the epidermis. It was indicated that its transport might be a key biosynthetic rate controlling point for the fluxes in secologanin production (Pan *et al.*, 2016). After being imported, loganic acid is methylated in the cytosol of the epidermis cells and subsequently further oxidized in the ER to yield secologanin (Pan *et al.*, 2016).

[2] The abbreviation IPP and DMAPP are based on the old names isopentenylpyrophosphate and dimethylallypyrophosphate instead of isopentenyldiphosphate dimethylallydiphosphate

2.3.2.3 The condensation of tryptamine and secologanin

The condensation of tryptamine (indole moiety) and secologanin (terpenoid moiety), catalyzed by strictosidine synthase (STR), generates strictosidine, which represents the central intermediate in MIA biosynthesis (Figure 2-6). Deglycosylation of strictosidine gives rise to a series of unstable intermediates for the biosynthesis of many different classes of MIAs Figure 2-4 (Thamm *et al.*, 2016).

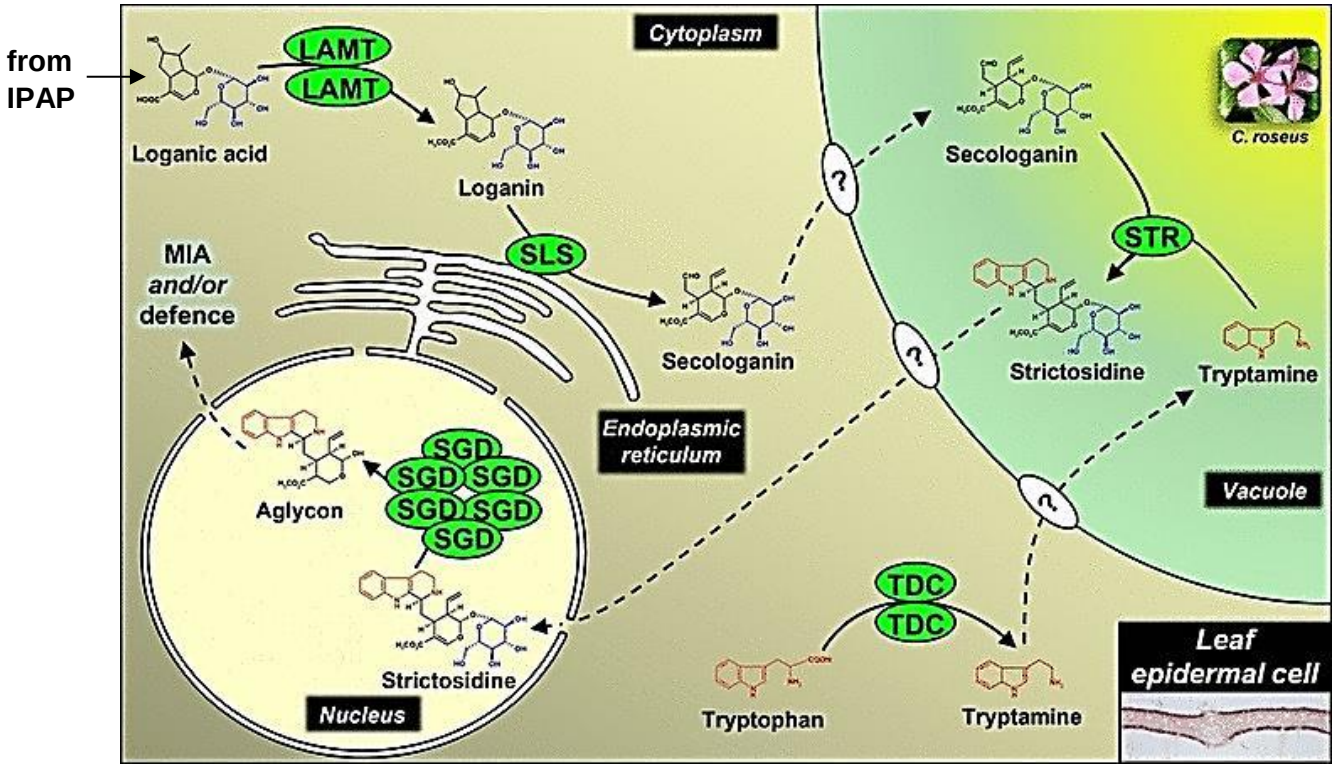


Figure 2- 6: Spatial model depicting the subcellular organization of the strictosidine biosynthetic pathway in an epidermal cell of *C. roseus* leaves. Loganic acid is translocated from the internal phloem associated parenchyma cells (IPAP) into the epidermal cells, where it is methylated a further converted to yield secologanin. '?' indicates the putative transportation system of tryptamine, secologanin, and strictosidine across the tonoplast. The number of repetitions of each enzyme name indicates whether it has been identified as a homodimer (LAMT or TDC) or multimer (SGD) (Guirimand *et al.*, 2011).

2.3.2.4 Final modifications

The unstable intermediates generated by the deglucosylation of strictosidine (Figure 2-4) are modified and converted to the corresponding final products. In this sense, a tremendously high amount of different structures is produced. Well investigated examples are the conversion of tabersonine to vindoline by the means of 7 modifying reactions or the coupling of vindoline and catharanthine to yield dimeric MIAs such as vinblastine (Costa *et al.*, 2008). Apart from oxidation and rearrangements also the loss of one or more C-atoms frequently contributes to the huge structural variation. Decarboxylation especially occurs at the carboxylic group in the

secologanin moiety (Dewick 2009). Yet, as these modifications generally are very specific for each species, no further details are outlined in this overview.

2.3.3 The alkaloid biosynthesis in *V. minor*

With respect to the alkaloid biosynthesis in *V. minor*, only limited information are available. Yet, due to the close genetic relationship to *C. roseus*, it is assumed that the same general biosynthetic pathway yielding strictosidine, resulting from the condensation of tryptamine and secologanin occurs also in *V. minor* (Figure 2-7). Verma et al. (2014b) proposed that strictosidine will be - catalyzed by a corresponding β-glucosidase (SGD) - deglucosylated and further converted to tabersonine. The latter one should represent the common intermediate for the major monoterpenoid indole alkaloids in *V. minor*. This assumption could be supported by incorporations of tabersonine in vincamine biosynthesis (Kutney *et al.*, 1971).

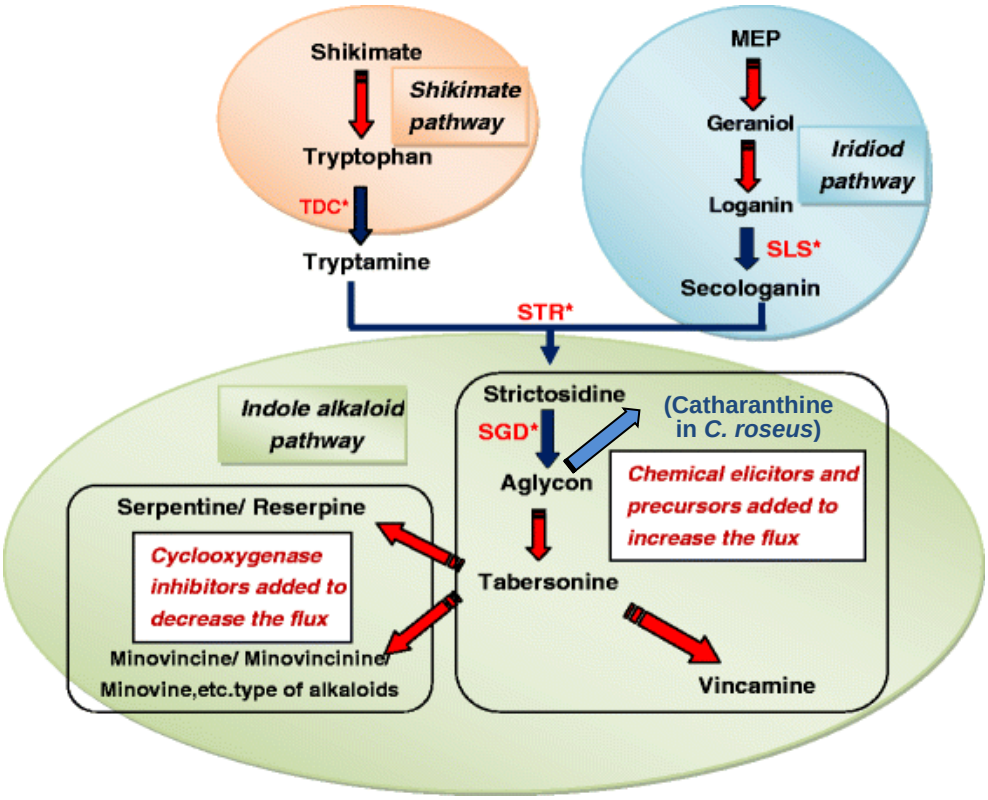


Figure 2- 7: Terpenoid indole alkaloid pathway in *V. minor* according to Verma et al. (2014b). *Red broken arrows* represent multi-step or uncharacterized reactions. **TDC:* tryptophan decarboxylase, *SLS:* secologanin synthase, *STR:* strictosidine synthase, *SGD:* strictosidine β-glucosidase. In *C. roseus* the right branch of the biosynthetic pathway leads to catharanthine via 4,21-dehydrogeissoschizine precursor (Guo *et al.*, 2013).

As the terpenoid skeleton in vincamine corresponds to type B (aspidospermine type, Figure 2-1; 2-8) like vincadifformine and vincatine, it was suggesting that vincamine biosynthetically should be derived from vincadifformine or from vincatine (Wenkert and Wickberg, 1965;

Saxton, 1983a; Kellner *et al.*, 2015). In this sense, it is worth mentioning that both alkaloids, vincatine (Doepke *et al.*, 1969) and vincadifformine (Plat *et al.*, 1962) co-occur with vincamine in *V. minor* (Herbert, 1983).

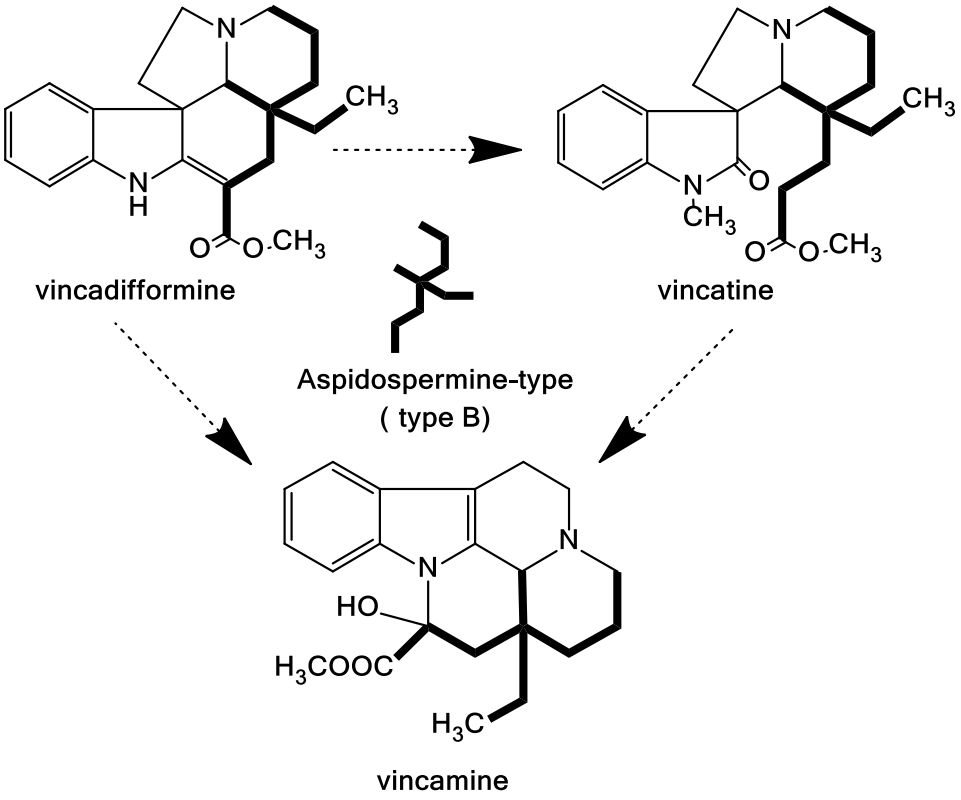


Figure 2- 8: Putative conversion of vincadifformine to vincamine. According to Mascavage et al. (2010), there are two possibilities to obtain vincamine from vincadifformine.

2.3.4 Regulation of indole alkaloids biosynthesis

Most of the monoterpene indole alkaloids (MIAs) reveal an important pharmacological relevance. Examples for their pharmacological effects are the anti-tumoral activity of the alkaloids from *C. roseus*, or the peripheral vasodilator activity for those from *V. minor* (Kutchan, 1995; Hasa *et al.*, 2013; Pan *et al.*, 2016). Since certain bioactive alkaloids often are present only in low concentrations in the corresponding plants, of many attempts had been made to increase their yields and to identify the factors regulating their synthesis and accumulation. Numerous studies applying various biotic and abiotic cues to monoterpenoid containing plants, elucidated that many different signaling pathways, including activating phosphorylation cascades, ion channel activity, accumulation of jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), ethylene, nitric oxide (NO) and reactive oxygen species (ROS) generation are involved in the regulation of the genes responsible for the alkaloid biosynthesis.

Since most of these data are derived from research employing *C. roseus*, in the following chapter, the corresponding insights are outlined in detail first for *C. roseus* solely. Subsequently,

on these coherences are merged with the limited data available for *V. minor*, in order to generate the scientific basis for the actual research presented in this thesis.

2.3.4.1 Regulation of alkaloids biosynthesis in *C. roseus*

MIA biosynthesis is highly complex and related to plant defense. It is controlled by a number of different signals including developmental cues, light, biotic and abiotic stress. Moreover, the synthesis of indole alkaloids in *C. roseus* strongly is affected by growth hormones (for review see (Zhao *et al.*, 2001a; El-Sayed and Verpoorte, 2007; Almagro *et al.*, 2015; Zhu *et al.*, 2015; Pan *et al.*, 2016). Yet, as this is extensively studied and not of major relevance for this thesis, the following review focuses growth regulators, which are known to impact the indole alkaloid synthesis. In this context, JA reveals the main attention due to its importance as a key regulator in the corresponding signaling pathways (DeGeyter *et al.*, 2012).

Jasmonate

Jasmonates (JAs) is frequently used as a collective term for jasmonic acid and their derivatives, like methyl jasmonate (MeJA) and other functionally active structural analogues. JAs are known to induce expression of genes involved in the biosynthesis of various natural products (e.g., De Geyter *et al.*, 2012), including the biosynthesis MIA (Gundlach *et al.*, 1992).

JAs are fatty acid derivatives synthesized by the so-called the octadecanoid pathway (Wasternack, 2007). Cleavage of membrane lipids by lipases yields α-linolenic acid, which is converted to the stable intermediate 12-oxo-phytodienoic acid (OPDA) by actions of 13-lipoxygenase (LOX2), 13-allene oxide synthase (AOS) and allene oxide cyclase (AOC). Reduction of this intermediate by OPDA reductase (OPR) and three subsequent β-oxidations yield jasmonate. Methylation of jasmonate yields the volatile hormone MeJA, and conjugation of jasmonate to isoleucine provides the most active form of JAs, jasmonoyl-isoleucine (JA-Ile; (Delker *et al.*, 2006; Thines *et al.*, 2007; Howe, 2018); Figure 2-9).

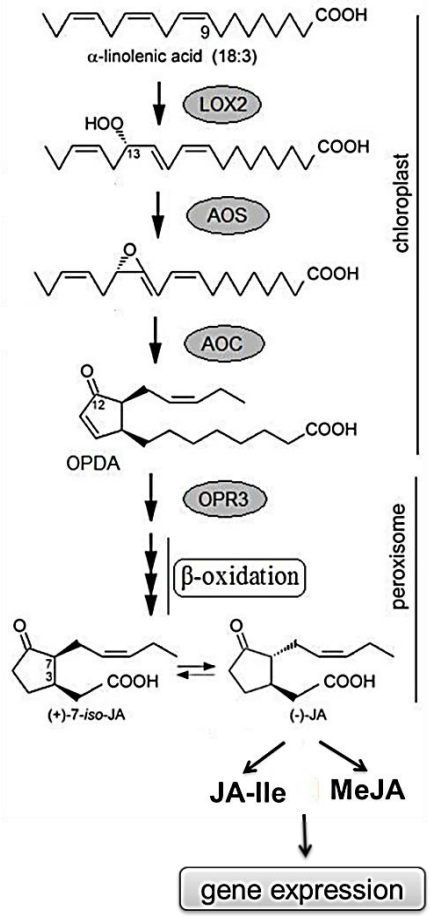


Figure 2- 9: Scheme of jasmonates biosynthesis pathway (Delker *et al.*, 2006).

Jasmonates are plant hormones that play key roles in signal transduction in many different responses to biotic or abiotic stress, including attacks by pathogens and herbivores, or drought stress (Creelman *et al.*, 1992; Gundlach *et al.*, 1992; Creelman and Mullet, 1997; Turner *et al.*, 2002; Riemann *et al.*, 2015). In most cases, jasmonate signaling leads to altered expression of genes, which encode various defense proteins or enzymes involved in the biosynthesis of natural products responsible for repelling herbivores or defending pathogens (Wei, 2010; Montiel *et al.*, 2011; Okada *et al.*, 2015). Moreover, jasmonic acid (JA) is also involved in the induction of leaf senescence and the corresponding catabolic processes (He *et al.*, 2002; El-Sayed and Verpoorte, 2005; Qi *et al.*, 2015).

One of the most prominent examples for the impact of methyl jasmonate (MeJA) on secondary metabolism concerns the indole alkaloids in *Catharanthus roseus* (Aerts *et al.*, 1994; Rijhwani and Shanks, 1998; Rodriguez *et al.*, 2003; El-Sayed and Verpoorte, 2005). Since jasmonates are involved in the induction and regulation of many different metabolic processes, clear and unequivocal cause-effect relationships cannot be observed in most cases. Indeed, when reviewing the related literature dealing with the impacts of MeJA on the synthesis and accumulation indole alkaloids in *C. roseus*, many - at first glance - contradictory findings are presented. For example, in hairy root cultures, the application of MeJA leads to a significant increase in the content of indole alkaloids and to a shift in their composition (Rijhwani and Shanks, 1998; Rodriguez *et al.*, 2003). In the same manner, also in mature *C. roseus* plants, the indole alkaloid content was increased by MeJA application (Naeem *et al.*, 2017). In contrast, Pan et al. (2010) showed that a MeJA treatment had no effect on the indole alkaloid accumulation in mature *C. roseus* leaves. Aerts et al. (1994) showed that MeJA effectively enhanced indole alkaloid biosynthesis in developing seedlings of *C. roseus* and that the enhancement of alkaloid biosynthesis decreased with age of the seedlings. In addition, the authors reported that the treatment of seedlings with MeJA induced changes in the content of tabersonine and caused the occurrence of additional peaks revealing a similar UV spectrum to tabersonine. However, due to preliminary results, the authors proposed that these alkaloids are neither hydroxy- nor methoxy- derivatives of tabersonine, which are known as the first two intermediates in the biosynthetic pathway from tabersonine to vindoline (Aerts *et al.*, 1994).

Further studies on the synthesis of MIAs employing *C. roseus* seedlings unveiled a high variability of the MeJA response (Wei, 2010). It was mentioned that the related impact of a MeJA treatment on the expression of genes responsible for alkaloid biosynthesis varied drastically - in magnitude as well as in timing. Accordingly, it is difficult to correlate any putative changes in indole alkaloid contents with alterations in the expression of specific genes

responsible for their biosynthesis (Wei, 2010). A compilation of the various MeJA effects on the MIA biosynthesis in *C. roseus* is outlined in Figure 2-10.

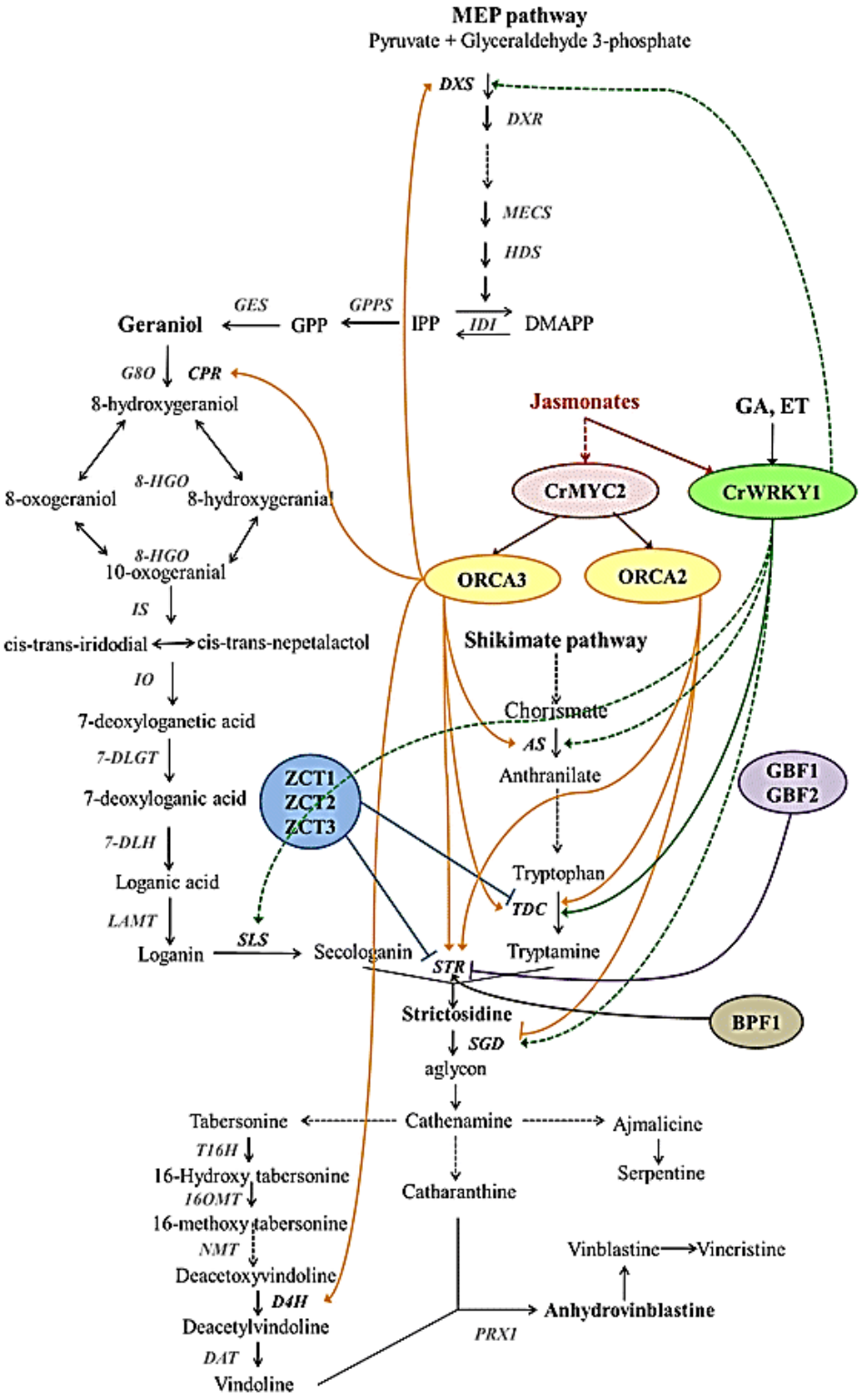


Figure 2- 10: Gene regulation scheme of the reported transcription factors involved in the MIA pathways. *Dashed lines* represent interactions that may be direct or indirect. *Solid lines* indicate potentially direct interactions. *Lines with arrows* represent transactivation, and *lines with bars* represent transrepression. *GA* gibberellic acid, *ET* ethylene. Enzymes in *black* represent to be regulated by reported transcription factors, enzymes in *grey* represent that their regulation has not been reported and remained unclear (Pan *et al.*, 2016).

Moreover, as further conjuncture is overcomplicating the described impacts of MeJA on the accumulation of MIA: when MeJA was applied to the detached leaves of *C. roseus*, the catabolism of alkaloids was strongly enhanced (El-Sayed and Verpoorte, 2005). All these – at least in part - inconsistent coherences clearly illustrate that in forthcoming investigations to elucidate any putative effect of MeJA on indole alkaloid biosynthesis, it is vital to consider thoroughly the physiological status of the plants. In this way, the effects of MeJA might be segregated from the effects of endogenous jasmonates produced due to natural stress situations or due to aging and senescence processes.

Salicylic acid

In addition to JA, salicylic acid (SA) is another important signaling molecule in plants. It has been well documented that SA is involved the defense responses related to the systemic acquired resistance (SAR) by activating defense-related genes (Thomma *et al.*, 2001; Hasanuzzaman *et al.*, 2017). Aerts et al. (1996) reported that the content of alkaloids is not affected when SA is applied to *C. roseus* seedling. In the same manner, Guo et al. (2013) did not find any effect on the MIAs when SA is applied to corresponding suspension cultures. In contrast, other authors reported the opposite effect: when SA is applied to seedlings or suspension cultures, respectively, the content of alkaloid increased (Godoy-Hernández and Loyola-Vargas, 1997; El-Sayed and Verpoorte, 2004). Similarly, the concentration of vinblastine, vindoline, and catharanthine significantly increased within a very short time period, when SA was applied to *C. roseus* plants at blooming stage (Pan *et al.*, 2010).

Hydrogen peroxide

H_2O_2 (Hydrogen peroxide) is a strong oxidizing agent, which is produced in the course of many reactions occurring in plants. The main source of H_2O_2 is related to the detoxification of superoxide radicals produced in the chloroplasts under drought stress by superoxide dismutase (Cruz de Carvalho, 2008). In general, the hydrogen peroxide is removed by ascorbate peroxidase (Asada, 1992). Moreover, huge amounts of hydrogen peroxide are involved lignification by creating the precursors for the radical chain reaction, and in photorespiration, resulting from the peroxidation of glycolate, tremendous quantities of H_2O_2 are generated, which are removed by the action of catalase (Slesak *et al.*, 2007; Saxena *et al.*, 2016).

In addition to these metabolic reactions, hydrogen peroxide also represents an important widespread signal molecule or signal transducer, respectively. It is involved in the induction of phytoalexin biosynthesis in the course of host-pathogen interactions, frequently denoted as

oxidative burst (Wojtaszek, 1997); or in numerous signalling events by interacting with other signal molecules, such as JA, ethylene, Ca^{++}, NO etc. (for review see (Saxena *et al.*, 2016)).

Various reports have been published, outlining that hydrogen peroxide is enhancing gene expression of various plant secondary metabolites (Wang *et al.*, 2011a). With respect to the MIAs only limited data are available. The concentration of indole alkaloids in leaves was positively elevated after exogenous application of H_2O_2 to *C. roseus* seedling. The time-course variations of alkaloid content showed significant correlation with redox state after H_2O_2 treatment (Tang *et al.*, 2009). Guo et al. (2013) mentioned that the application of H_2O_2 enhances the content of tabersonine in the cell suspension culture of *C. roseus*, while the biosynthesis of catharanthine and serpentine was significantly decreased. On the contrary, when the concentrations of hydrogen peroxide was increased further on, the content of tabersonine within the cells decreased, maybe as result of cell damage by high concentrations of H_2O_2. Alternatively, it could be assumed that tabersonine might be oxidized, since it is known that MIAs can act as an efficient substrate for peroxidase in *C. roseus* (Tang *et al.*, 2009). The corresponding decrease in the concentration of H_2O_2 due to the peroxidation of MIAs is discussed by Tang et al. (2009) as contribution to antagonizes the oxidative environmental stress (Xu and Dong, 2005; Tang *et al.*, 2009; Matsuura *et al.*, 2014).

2.3.4.2 Regulation of alkaloids biosynthesis in *V. minor*

Based on the comprehensive knowledge of the regulation of alkaloid biosynthesis *C. roseus* outlined above, Verma and coworkers (2014b) used several approaches to elaborate information on the regulation of the biosynthesis of MIAs in *V. minor*. According to the biosynthetic pathway outlined in Figure 2-7, tabersonine represents an important branching point. The authors applied naproxen to hairy roots cultures of *V. minor*, which is known to modulate the serpentine biosynthesis in *C. roseus* by inhibiting the cyclooxygenase (Guo *et al.*, 2011). In contrast to the findings in *C. roseus*, where naproxen – as expected – lead to a decrease in the serpentine concentration (left branch outlined in Figure 2-7) accompanied by an increase in the catharanthine concentration (right branch). Yet, in hairy root cultures of *V. minor*, naproxen had no effect, neither on total alkaloid nor on vincamine content (Verma *et al.*, 2014b). Unfortunately, the authors used a system, in which no vincamine was present even in the control and thus no "right branch" was present. Nevertheless, some insights in the regulation of MIAs could be elaborated: hydrogen peroxide caused a significant enhancement of total alkaloid content. Obviously, H_2O_2 is involved also in *V. minor* in the regulation of MIAs.

2.3.5 Localization of indole alkaloids in *V. minor* and *C. roseus*: revision of the alleged surface localization

Plants are well known to secrete an array of secondary metabolites onto their surfaces (e.g.; LoPresti, 2016). According to our basic understanding of chemical ecology, these substances represent the first chemical barrier against herbivory or attack by fungal or bacterial pathogens. Apart from waxes, these exudates may contain unusual fatty acids and triterpenes, e.g., the leaf of the bitter olive *Olea europaea* (Oleaceae) is heavily coated by oleanolic acid, which acts as a potent barrier (Kubo and Matsumoto, 1984). Unfortunately, only a little evidence for the secretion of alkaloids onto the plant surface is available. Just recently, it was reported that water-soluble pyrrolizidine alkaloids are present at the surface of *Senecio jacobaea* (Vrieling and Derridj, 2003). Although the levels found were small compared with those within the leaves, the authors deduce that the alkaloids had been exuded.

Concerning the occurrence of MIAs on the leaf surface, various studies for *C. roseus* and *V. minor* had been published. Roepke et al. (2010) reported that catharanthine is present at the leaf surface of several *Catharanthus* species, whereas vindoline, anhydrovinblastine, and other MIAs would be accumulated only within leaf cells. Indeed, catharanthine was present in so-called chloroform surface extracts, whereas chlorophyll and the other MIAs were absent - as well as chlorogenic acid, which the authors claimed to be a marker for symplastic leaf epidermis metabolites (Roepke *et al.*, 2010). Unfortunately, these deductions are false, since chloroform – even when only a short time is applied – will destroy the plasmalemma of the epidermal cells and change the localization of substances (Bewick *et al.*, 1993; Derridj *et al.*, 1996). Consequently, catharanthine will also be present in the chloroform extract when it is localized within the epidermis (symplastically). Moreover, chlorogenic acid, the proposed symplastic marker, used by the authors, is insoluble in chloroform (Lu *et al.*, 2004) and accordingly will not be present in the organic phase even when the cells are disrupted. Yet, the fact that catharanthine is present in the extracts whereas vindoline, anhydrovinblastine are not, points to a differential spatial localization of the different MIAs. It seems to be very likely that catharanthine is present in the epidermal layer, whereas the other MIAs predominantly are located within the mesophyll. In this context, much more research employing sound and reliable methods are required. The situation corresponding the localization of MIAs in *C. roseus* becomes even more complex, since by using both, imaging mass spectrometry (MS) and single-cell MS, (Yamamoto *et al.*, 2016) shown that – at least in the stem of *C. roseus* – all MIAs are accumulated in idioblast and laticifer, whereas the only catharanthine is also present in the epidermal layer. The authors did not detect any substances in the wax layer.

Moreover, Demessie et al. (2017) postulated that MIAs, vincamine, akuammicine, lochnericine, minovincinine and vincadifformine are secreted on leaf surfaces of *V. minor*. Unfortunately,

also, in this case, the same doubtful method had been applied. Accordingly, there is no evidence for an apoplastic occurrence of MIAs in *V. minor*. It is worth mentioning that pattern of surface and whole leaves extract of indole alkaloid mentioned by (Demessie *et al.*, 2017) did not match at all to earlier published data of chemistry of leaves of *V. minor* (Proksa and Grossmann, 1991; D'Amelio Sr. *et al.*, 2012; Hasa *et al.*, 2013) especially vincamine is present only in minor amounts. Overall, the data presented in this publication have to be queried.

According to the alleged occurrence of MIAs on the surface of *V. minor* leaves, it was argued that corresponding carrier systems must be present, which should be responsible to exude the alkaloids into the apoplastic cuticle. Based on transporter assays employing yeast cell, in which the putative transporters (CrTPT2) had been heterologously expressed, efflux velocities for vincamine and vincadifformine had been analyzed (Demessie *et al.*, 2017). The authors postulated that the secretion mechanism of the MIAs is mediated by an ABC transporter. However, as in many cases, alkaloids are re-located within the plants (Yazaki *et al.*, 2008; Pakdeechanuan *et al.*, 2012). In this context, it has to be considered that these transporters have to efficiently bind and translocate the alkaloid cations, whereas the free base diffuses freely through biomembranes. In fact, using isolated vacuoles from *C. roseus* leaves, Carqueijeiro et al. (2013) demonstrated that catharanthine and vindoline were accumulated in the vacuoles of mesophyll cells. The authors postulated that this accumulation is driven by a specific proton antiport system, which dependents on the transtonoplast pH gradient generated by V-H^+-ATPase from leaves. In addition, Yamamoto et al. (2016) reported that the complex distribution pattern of the MIA intermediates in *C. roseus* suggested that the enzymes responsible for synthesis are located in one cell type, but that the enzyme products are then moved to other cells. Furthermore, the former authors tried to explain these contradictions in the observed results by assuming that different cultivars with different developmental stages, and growth conditions, may likely affect the compartmentalization expression of the MIAs pathway. In consequence, the occurrence of a transporter for MIAs does not prove that the alkaloids are exuded. Based on the coherences outlined, it seems to be very likely that these transporters are required for translocation of these substances out of the vacuoles.

2.4 Non-alkaloidal compounds of *V. minor*

In comparison to the extensive literature on MIAs, only very little work has been done concerning the isolation and identification of non-alkaloidal natural products from *V. minor*. Up to now, predominantly various phenolic compounds are reported to occur; apart from several typical flavanoids (various kaempferol and quercetin glycosides) also chlorogenic acid and some dihydroxybenzoic acid derivative had been reported (Raynaud *et al.*, 1970; Nishibe

et al., 1996). Moreover, ursolic acid and various steroids (β-sitosterol, triacontane; (Le Men and Hammouda, 1956) also lignans such as lirioresinol B, and anthocyanin (Tatsuzawa, 2015) had been detected to occur in the little periwinkle (Garnier *et al.*, 1975).

2.5 Biological activity of *V. minor*

2.5.1 In folk medicine

In folk medicine, little periwinkle is used internally for circulatory disorders, cerebral circulatory impairment and support for the metabolism of the brain. It is also used internally for loss of memory, hypertension, cystitis, gastritis, and enteritis, diarrhea, raised blood sugar levels and to help weaning. Periwinkle is used externally for sore throats, nose bleeds, bruising, abscesses, eczema and to stop bleeding. In Homeopathy extracts of *V. minor* are used to treat weeping eczema and bleeding mucous membranes (Fleming, 2004).

2.5.2 Vincamine and derivatives as cerebrovascular and neuroprotective agents

Vincamine, frequently denoted as ‘‘mother compound’’ of cerebrally active eburnamine derivatives, was first isolated from the leaves of *Vinca minor*. The first pharmacological studies were done by several research groups in the 1960s (Raymond-hamet, 1954; Quevauviller *et al.*, 1955). These studies demonstrated the beneficial effect of vincamine on circulation by decreasing arterial blood pressure, cardiac output, and heart rate. Yet, the extensive investigation revealed that vincamine strongly improved cerebral functions, rather than to lower the blood pressure. In consequence, vincamine was introduced for the treatment of cerebral insufficiencies in Europe (Vas and Gulyás, 2005). Subsequently, research focused on the mechanism of action of this alkaloid. Furthermore, based on structural modification and the identification of further MIAs, more potent derivatives had been searched. Out of several thousand derivatives investigated, only three reached the pharmaceutical market. These compounds reveal the significant beneficial effect on brain circulation and neuronal homeostasis. The common structural features of these three molecules are given by the *cis*-fused ring system of vincamine, and the esterification of the carbonyl function at C-16 (Figure 2-11).

Until now, various molecular targets of vincamine and its synthetic derivative (vinpocetine) are known, the phosphodiesterase-1 (PDE-1), the voltage-dependent Na^+ and Ca^{2+} channels, and glutamate receptors. In particular, the inhibition of PDE-1 seems to be responsible for the positive vascular effects, for the improvement in cerebral circulation, and for the beneficial effect on platelets (Tárnok *et al.*, 2008; Nemes, 2010; Medina, 2011).

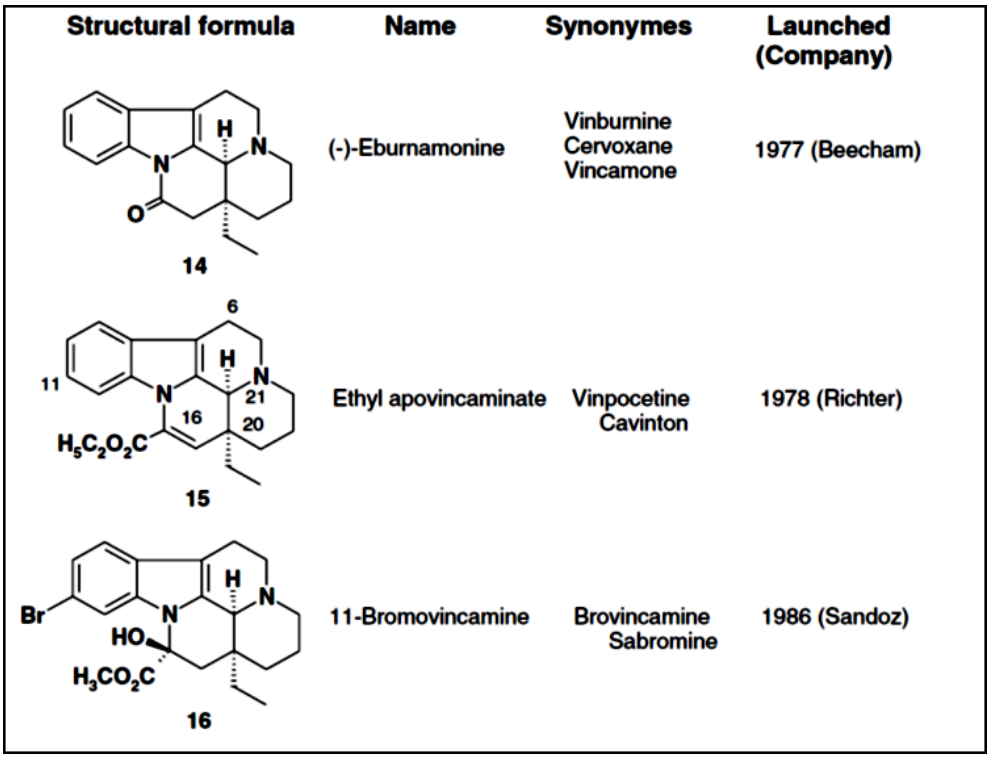


Figure 2- 11: Structures of clinically used vincamine derivatives according to Nemes, (2010).

Abdel-Salam et al. (2016) reported that the low therapeutic doses of vincamine and vinpocetine present in several nootropic drugs reveal antioxidant and neuroprotective effects. Higher doses are thought to have pro-oxidant and pro-inflammatory properties. This is underlined by the finding that vincamine protects PC12 cells against hydrogen peroxide-induced oxidative stress. The underlying molecular mechanism was elucidated by (L Wu *et al.*, 2016) who demonstrated an up-regulation of superoxide dismutase and an activation of the phosphatidylinositol-3 kinase/protein kinase B pathway. Other studies also provide the evidence that vinpocetine may exert protective effects via modulating oxidative stress and apoptosis in Mn-induced neurodegeneration in NE-4C cells (Bora *et al.*, 2016). In addition, further studies showed that vinpocetine possessed anti-inflammatory activity by inhibiting of NF-κB activation and subsequent up-regulation of pro-inflammatory cytokines (Ghosh *et al.*, 1998; Jeon *et al.*, 2010; Medina, 2010), while others demonstrated a novel role of vinpocetine to decrease lipid accumulation and atherosclerosis formation in mice (Cai *et al.*, 2013).

Antioxidant activity of alkaloids could have implications for the better understanding of plant protection mechanisms against stresses and to find out additional pharmacological uses for MIAs. For instance, vincamine of *V. mino*r, used as a neuroprotective agent, caused a decrease in the iron content of rat brain, possibly decreasing oxidative stress caused by the accumulation of this metal during neurodegenerative processes (Fayed, 2010; Han *et al.*, 2017). Additionally, the semi-synthetic MIA vinpocetine (produced from vincamine) inhibited the free radical formation and lipid peroxidation in brain striatum-isolated nerve endings (Herrera-Mundo and

Sitges, 2013). These studies could state the proposed protective role of vincamine in the plant against ROS produced under different stress situations.

2.5.3 The cytotoxic and antimicrobial activities of *V. minor*

Numerous studies have been carried out to investigate the cytotoxic and antimicrobial activities of the *V. minor* plant extracts. The corresponding results are displayed in Table 2-2.

Table 2- 2: Cytotoxic and antimicrobial activities of *V. minor.*

Compound/ extract	Activity	References
Vincarubine	Cytotoxic effect against leukemia cells	(Proksa *et al.*, 1986; Sturdíková *et al.*, 1986; Proksa *et al.*, 1988)
Vincadifformine	The cytotoxic activity against leukemia cells is higher than that of vinblastine	(Sturdíková *et al.*, 1986; Khanavi *et al.*, 2010)
Vincamine	Antifungal and antibacterial activity	(Ozçelik *et al.*, 2011)
Fractions of alkaloid extract	Cytotoxic effects on breast ductal and colon carcinoma, and colorectal adenocarcinoma cells	(Khanavi *et al.*, 2010)
Aquatic, acetone and ethyl acetate extracts	Toxic effects against *Rhodotorula* and *Candida*.	(Yildirim *et al.*, 2013; Grujić *et al.*, 2015)

2.6 Completion

The brief review of the literature presented above expounds that - in contrast to the rich literature on the biosynthesis of indole alkaloids and its regulation in *C. roseus* –corresponding information for *V. minor* is limited. Although numerous studies outlined quite different compositions of indole alkaloids of *V. minor*, in all cases, the major alkaloid in the leaves was reported to be vincamine (Proksa and Grossmann, 1991; D'Amelio Sr. *et al.*, 2012; Hasa *et al.*, 2013). As known for other alkaloids, the content of vincamine varies largely depending on growth and climatic conditions, the season, and putative stress situations (Boyadzhiev *et al.*, 2002). Accordingly, in this study, which should generate new approaches for phytochemical drug discovery, only mature *V. minor* plants of defined physiological status will be used to investigate the effect of MeJA treatment and of application of other growth regulators on the composition of alkaloids. Apart from such modulation of the composition of pharmaceutically active compounds by applying growth regulators, in principle, also gene technological approaches, i.e., by modifying certain gene responsible for the secondary metabolism or its regulation respectively, could be accomplished. Yet, such strategy is not in the centre of focus of this thesis.

Chapter 3: Materials and methods

3.1 Plant material and chemicals

3.1.1 Plant material

Vinca minor L. mature plants at the blooming stage were purchased from a commercial market garden (Brennecke GmbH, Braunschweig, Germany).

3.1.2 Chemicals

Name	Purity	Company
Acetic acid	HPLC	Fisher Scientific, Loughborough, UK
Acetonitrile	HPLC	VWR, Leuven, Belgien, Germany
Ammonium acetate	HPLC	Fluka, Steinheim, Germany
Ammonium hydroxide	25%	Roth, Karlsruhe, Germany
Catharanthine	≥ 95%	Sigma-Aldrich, Steinheim, Germany
$CHCl_3$-*d*	99.96 %	Deutero, Kastellaun, Germany
Chloroform	Analytical grade	Fisher Scientific, Loughborough, UK
DMSO-d_6	99.96 %	Deutero, Kastellaun, Germany
Formic acid	99% LC/MS	VWR, Leuven, Belgien, Germany
Hydrochloric acid	37%	Roth, Karlsruhe, Germany
Hydrogen peroxide	30%	Roth, Karlsruhe, Germany
Methanol	HPLC	VWR, Leuven, Belgien, Germany
Methyl jasmonate	95%	Sigma-Aldrich, Steinheim, Germany
Naproxen	≥ 98%	Sigma-Aldrich, Steinheim, Germany
Resveratrol	≥98 %	Roth, Karlsruhe, Germany
Salicylic acid	≥99 %	Sigma-Aldrich, Steinheim, Germany
Strychnine	98%	Sigma-Aldrich, Steinheim, Germany
Triethylamine	HPLC	Fisher Scientific, Loughborough, UK
Trifluoroacetic acid	99.9%	Roth, Karlsruhe, Germany
Triton X- 100	laboratory grade	Sigma-Aldrich, Steinheim, Germany
Vincadifformine	95%	Carbosynth, UK
Vincamine	≥97.0%	Sigma-Aldrich, Steinheim, Germany
Vindoline	≥98.0%	Sigma-Aldrich, Steinheim, Germany

3.2 Equipment and parameters

- **High-Performance Liquid Chromatography (HPLC)**
  - Pump: YL9110 Quaternary Pump
  - Degasser: YL9101 Vacuum Degasser
  - Injector: Autosampler Midas Spark Holland, Model 830
  - Column oven: YL9130 Column Compartment
  - Detector: YL9160 PDA Detector
  - Software: YL-Clarity, Chromatograph Data System, Vers. 4.0.4.987

- **Liquid chromatography-mass spectrometry (LC-MS)**
  - HR-ESI-MS and LC-ESI-MS/MS experiments were recorded using a Bruker maxis HD UHR-TOF mass spectrometer with Apollo II ion funnel ESI Electrospray source. A UHPLC-system (Ultimate3000RS from Dionex/Thermo) was used for separation.
  - The program used for analysis of LCMS data: Bruker Compass Data Analysis 4.2 (Bruker Daltonic GmbH, Bremen, Germany). Included Function (Find Molecular Features) was used for the quantification. The mass spectrum calculation was performed with Line Spectra.
  - Mass spectrometric condition: The mass spectrometer was operated in positive electrospray ionization mode and spectra were recorded by scanning the mass range from 50 to 1500 m/z in both MS and MS/MS modes. Nitrogen was used as drying, nebulizing and collision gas. The drying gas flow rate was 9.0 L/min. The dry gas temperature was set to 200 °C and nebulizer pressure at 4.0bar. For the MS/MS analysis, the collision energy was ramped between 17 and 55 eV, depending on the fragmented *m/z* and the charge state of the isolated mother ion.

- **Nuclear Resonance Spectroscopy (NMR)**

NMR spectra were recorded using a Bruker Avance III 700 spectrometer with a 5 mm TCI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz) and an Avance III 500 (^{1}H 500 MHz, ^{13}C 125 MHz) spectrometer (performed by Dr. Frank Surup, Microbial Drugs Department, Helmholtz Centre for Infection Research, Braunschweig).

- **Polarimeter**

Optical rotations were determined using a Perkin-Elmer 241 Polarimeter, USA

- **UV-Vis spectrophotometer**

UV spectra were recorded using a Shimadzu UV-Vis spectrophotometer UV-2450, Japan

- **Freeze drying**
  - Device: Lyovac GT2, Finn-Aqua, Germany
  - Pumpe: Trivac, Leybold
- **Ball mill (Retsch MM200), Germany**
- **Turbo Vap LV evaporator (Zymark), USA**
- **Rotary Evaporators (Heidolph type VV2000), Germany**
- **Ultrasonic bath (Bandelin Sonorex), Germany**
- **Hettich Rotixa/RP Centrifuge, Germany**
- **Eppendorf centrifuge (5415 D), Germany**
- **Vortex-Genie 2, Scientific industries, USA**

3.3 Extraction and isolation of indole alkaloids from *Vinca minor* control and MeJA-treated leaves

3.3.1. Plant growing conditions

Vinca minor mature plants at the blooming stage were purchased from a commercial market garden (Brennecke GmbH, Braunschweig, Germany). The individual plants were transferred into pots (15 cm in diameter) containing a soil-sand mixture (3:1), prepared from commercially available substrate (Floragard, Oldenburg, Germany) and sand. During acclimation phase, the soil moisture of all pots was adjusted to 25–30%, and the temperature range during the experiment was 15–20 °C. Plants were grown in the garden of the Institute of Plant Biology of TU-Braunschweig. Plants were kept under rain shelter to prevent water input from the rain Figure 3-1.

Figure 3- 1: *Vinca minor* plants at the beginning of the experiment.

3.3.2 Application of methyl jasmonate

For application of MeJA, plants were taken out of the growth area and transferred into an open area in about 20 m distance from the control plants and sprayed with MeJA solution (0.5 mM, containing 0.2% Triton X; (Kleinwächter *et al.*, 2015)) on both sides of the leaves, until liquid dripped from the leaves (15 mL/plant), MeJA was applied two times during the experiment (on days 1 and 4). Treated plants were harvested after 9 days.

3.3.3 Sampling

Arial parts of experimental plants were cut from their roots. The leaves were separated from the stems. The plant material was shock frozen in liquid nitrogen and stored at −20 °C until further extraction and analyses. For preparative work, above ground parts of 6 plants were cut from their roots and shock-frozen and freeze-dried in liquid nitrogen.

3.3.4 Analytical methods

For determination of alkaloids, 300 mg of freeze-dried leaves was ground to a fine powder using a ball mill (RetschMM200). Then a sample was extracted with methanol (3 × 2 mL) in an ultrasonic bath at 50 °C for 30 min. After centrifugation (10 min at 2,500 x g), the combined methanolic (MeOH) extracts were evaporated using Zymark Turbo Vap evaporator, USA, at 50 ^{0}C. The dried MeOH extract was further extracted with 2 mL and subsequently with 1 mL of 3% HCl (twice) in an ultrasonic bath at 50 ^{0}C for 30 min. After centrifugation (10 min at 4000 rpm), the combined aqueous extracts were cooled at 4 °C and treated with 25% ammonium hydroxide (NH_4OH) to reach pH 8-9. Alkaloids were extracted three times with $CHCl_3$ (3 × 2 mL). Combined $CHCl_3$, extracts were dried (constant weight) and then dissolved in MeOH (1 mL). Before performing LCMS analysis, samples were filtrated (0.45 µm, Spartan).

The LC-MS separation was performed on a Kinetex C_{18}-column (1.7 µm, 100 Å, 150 × 2.1 mm) from Phenomenex, using a gradient system. Solvent A was water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The gradients used were 0 min, 1% B; 5 min, 15% B; 42 min, 30% B; and 50 min, 100% B. The flow rate was 300 µL/min. The overall runtime was 60 min at a column temperature of 40 °C.

3.3.5 Extraction and isolation of pure compounds from MeJA-treated plants

Ten grams of freeze-dried aerial parts were extracted as previously mentioned to obtain approximately 150 mg of crude alkaloids. The alkaloids were dissolved in methanol and separated by HPLC. Separation was performed on a VP 250/10 Nucleosil 100-5µ RP-18 semi-preparative column using a gradient system. A: acetonitrile; B: ammonium acetate (15 mM),

containing 0.2% triethylamine, adjusted with acetic acid to pH 4. The gradients used were 0 min, 15% A, 85% B; 7 min, 20% A, 80% B; 42 min, 40% A, 60% B; 47 min, 80% A, 20% B; 50 min, 15% A, 85% B; and 60 min, 15% A, 85% B. The flow rate was 3 mL/min. The injection volume was 100 µL by manual injection. Alkaloids were monitored using a photodiode array (PDA) detector at 254, 280, and 330 nm. Fractions were manually collected and combined according to the observed peaks. Eight fractions were collected (Figure 3-2; 3-3): fraction 1 afforded compound **9** with a retention time t_R= 10.5 min; fraction 2, compound **6** at t_R= 16.8 min; fraction 3, compound **1** at t_R= 21.8 min; fraction 4, compound **2** at t_R= 25.9 min; fraction 5, compound **7** at t_R= 29.4 min; fraction 6, compound **5** at t_R= 30.2 min; fraction 7, compound **3** at t_R= 35.1 min; and fraction 8, compound **4** at t_R= 39.3 min.

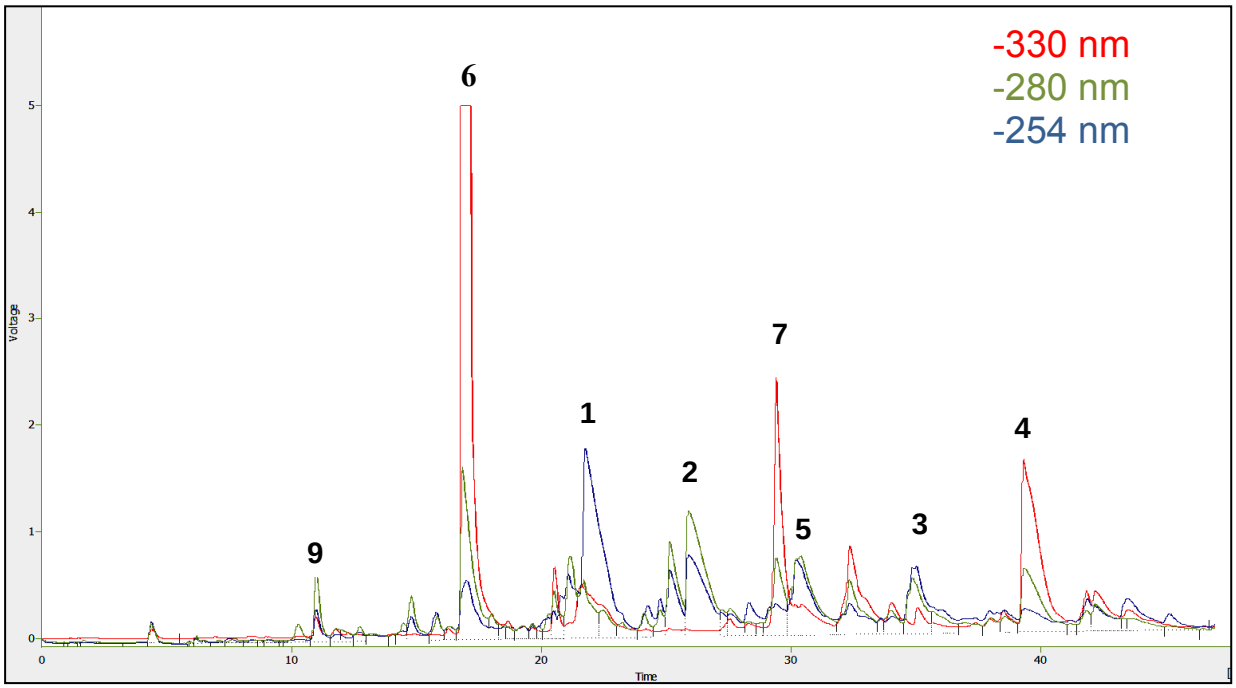


Figure 3- 2: Semi-preparative HPLC separation chromatogram of the major indole alkaloids determined in *V. minor* treated with MeJA.

Due to the presence of NH_4OAc and Et_3N, the exact weight of the substances could not be determined at this stage of isolation. Thus, to eliminate the NH_4OAc and Et_3N and for further purification, the freeze-dried samples were dissolved in MeOH and again purified by HPLC. Solvent A: acetonitrile, 0.1% formic acid or TFA; B: H_2O with 0.1% formic acid or TFA. The gradients used were 0 min, 15% A; 5 min, 20% A; 42 min, 40% A; 45 min, 80% A. The flow rate was 3.5 mL/min. After freeze-drying, the alkaloids were weighed (approximate yield compound **9**= 1 mg, **6** = 3 mg, **1** = 3 mg, **2** =3 mg, **7** = 1.5 mg, **5** = 3 mg, **3** = 2 mg, **4** = 2 mg) and analysed (Figure 3-3).

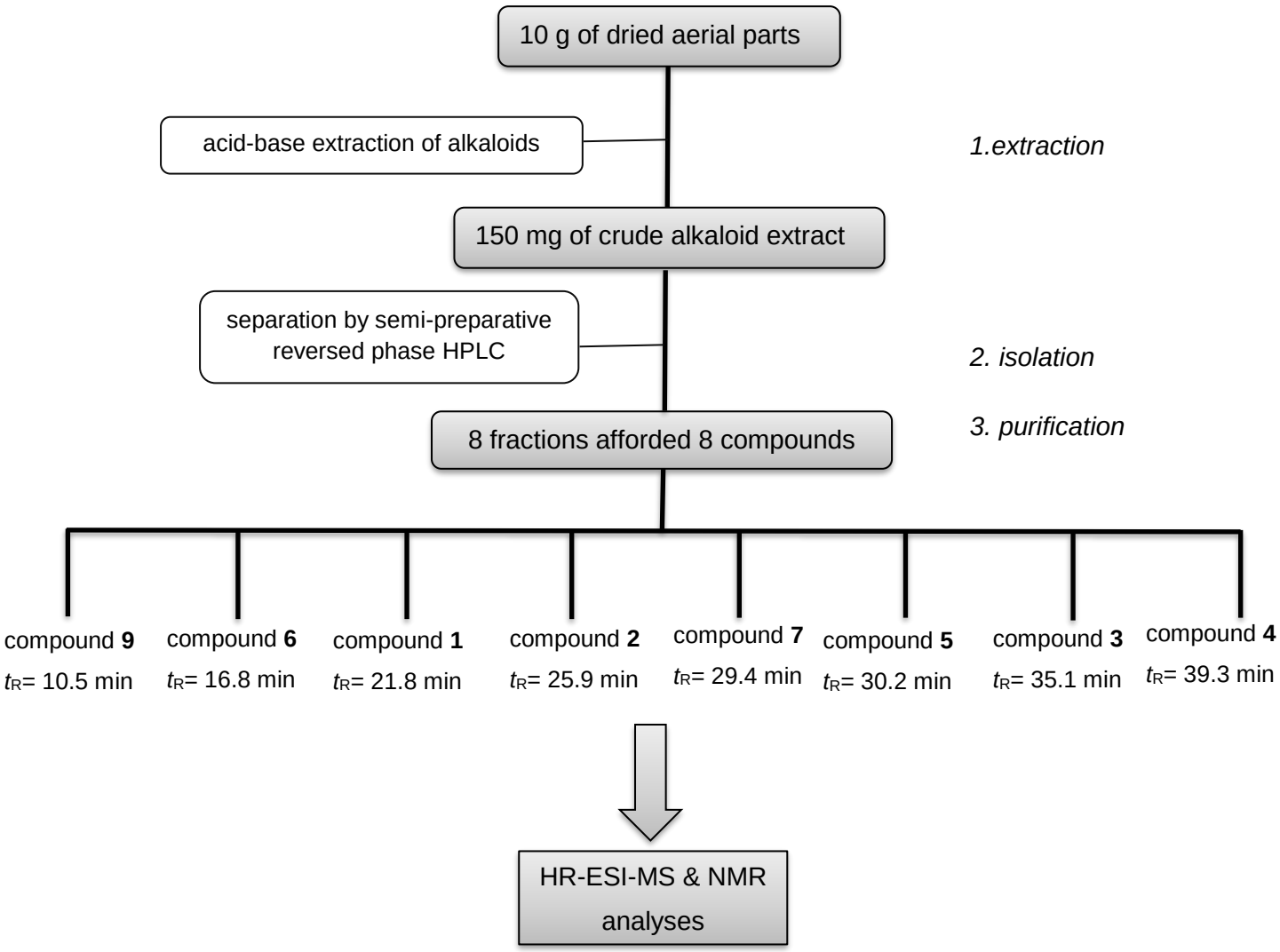


Figure 3- 3: The main steps of extraction and isolation of MIAs from *V. minor* leaves.

3.4 Application of different growth regulators on *V. minor* leaves

3.4.1 Plant material

Vinca minor mature plants at blooming stage were purchased from a commercial market garden (Brennecke GmbH, Braunschweig, Germany). Girre (1971), and Boyadzhiev et al. (2002) reported that the two highest concentration of vincamine in the herb which coincides with the periods of flowering and fruits ripening in April and in September, respectively. In addition, *V. minor* is more resistant to cold, and its optimal growth environment temperature is 10 °C (Chen *et al.*, 2017). Thus, the experiment was established in April 2017. The individual plants were transferred into pots (15 cm in diameter) containing a soil prepared from the commercially available substrate (Floragard, Oldenburg, Germany). During acclimation phase, the soil moisture of all pots was adjusted to 25–30%. The medium temperature during the experimental period was 8 °C with a minimum of 4.4 °C and a maximum of 11.5 °C. Then, the flowers were

removed from all plants 2 weeks before the starting of the experiment. Plants were grown in the garden of the Institute of plant biology of TU-Braunschweig (Figure 3-4). Plants were kept under rain shelter to prevent water input from the rain.

Figure 3- 4: *Vinca minor* plants during the experiment.

About two weeks later, the following treatments were set up in comparison to a control (Figure 3-5).

3.4.2 Application of methyl jasmonate (MeJA), salicylic acid (SA) and hydrogen peroxide (H_2O_2)

Application of MeJA was performed by using (0.5 mM) solution. For adequate solubilization, solutions contained 0.2% Triton X was used according to Kleinwächter et al. (2015). Application of SA and H_2O_2 were performed by using a 0.5 mM and 20 mM solutions respectively, which also contained Triton X (0.2%). For all approaches, plants were sprayed with 15 mL of the solutions (Figure 3-5). In the case of MeJA treated plants were taken out of the growth area and transferred in an open area about 30 m distance from the control plants and sprayed with MeJA solution on both sides of leaves until liquid dripped from the leaves.

Six plants were collected before the application of the treatment. After one day of the application of treatments, the first harvest was performed. The remaining plants were sprayed again after 3 days and plants were harvested at fourth, eighth and fourteen days from the beginning of the experiment. For each treatment (control, Triton X100, 0.5 mM MeJA or SA, and 20 Mm H_2O_2), six replicates were sampled at each harvest. Accordingly, in total, 96 pots have been grown.

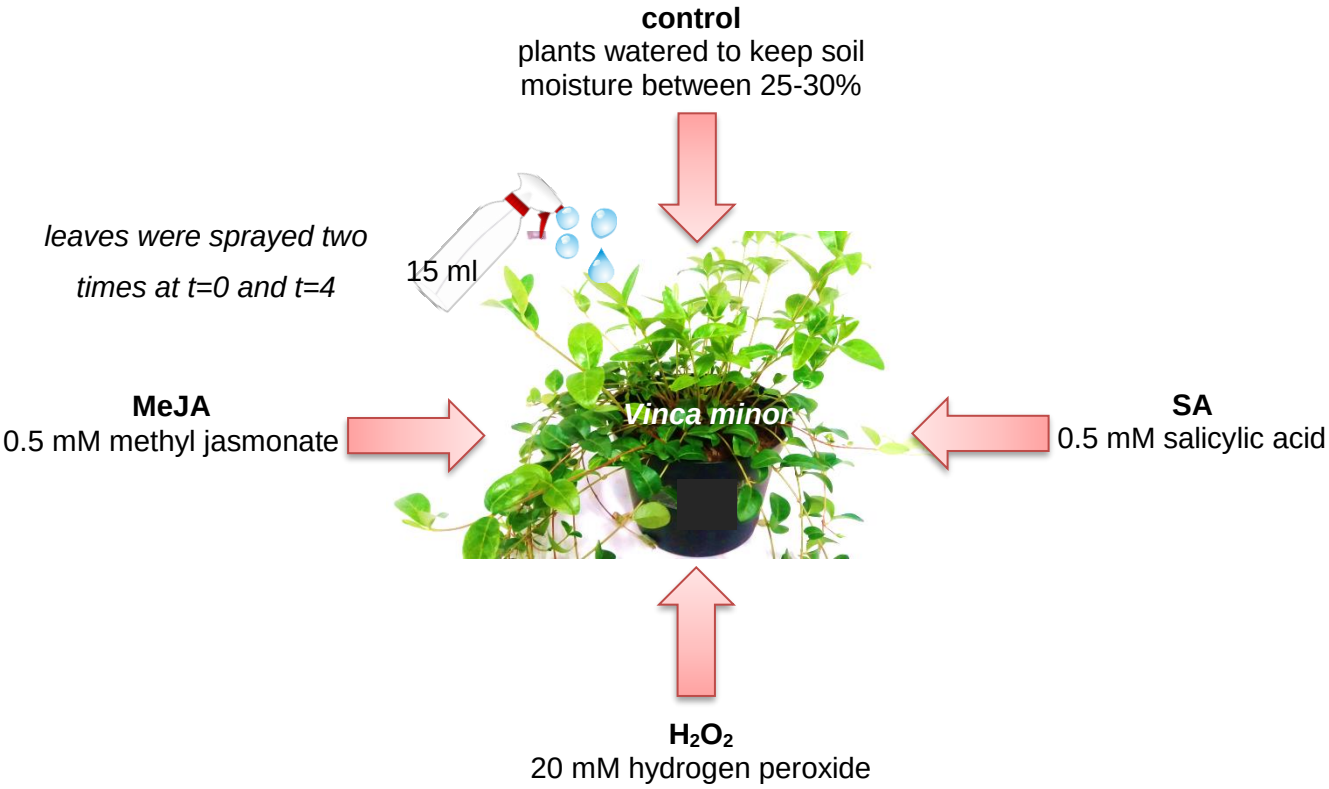


Figure 3- 5: *V. minor* leaves treated with different growth regulators. Spray applications were applied two times during the experiment. Sampling dates: 18.04.2017, 21.04.2017, 25.04.2017 and 1.05.2017.

3.4.3 Sampling

Arial parts of experimental plants were cut from their roots. Leaves were separated from stems and divided into young and old leaves (Figure 3-6). The plant material was shock-frozen in liquid nitrogen and stored at −20˚C until further extraction and analyses, respectively. Moreover, to calculate the average gain of biomass per leaf during the maturation: the weight of six groups of young and old leaves (each group contains eight leaves) was measured before and after freeze drying.

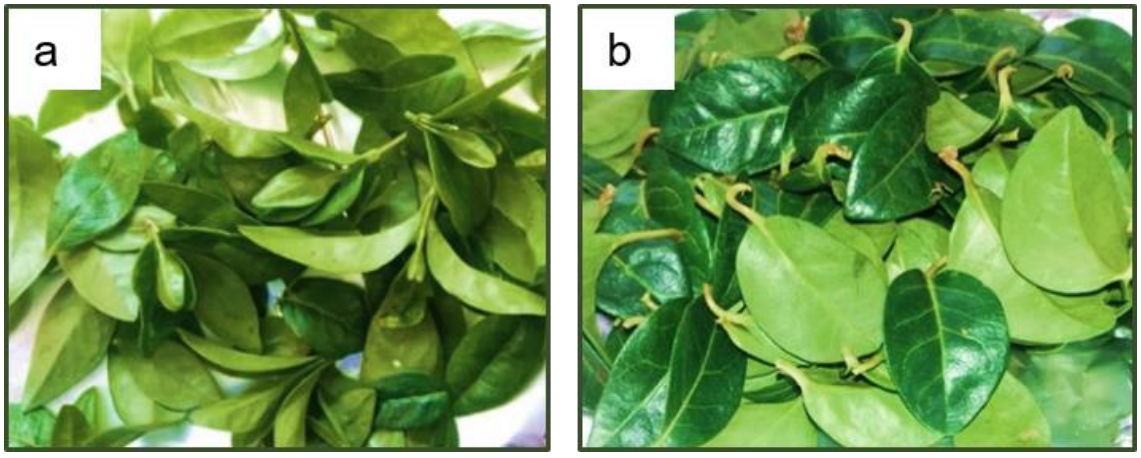


Figure 3- 6: Leaves were separated and divided into a) young leaves and b) old leaves.

3.4.4 Extraction and quantification of indole alkaloids in *Vinca minor*

- *Extraction of indole alkaloids*

For the determination of alkaloids, 100 mg of freeze-dried leaves were ground to fine powder using a ball mill (RetschMM200). Subsequently, the sample was extracted once with 1 mL of MeOH containing 300 mg/L strychnine as internal standard (IS) and then with 1.5 mL pure MeOH (twice) in an ultrasonic bath at 50°C for 30 min each and last time was kept overnight for complete extraction. After centrifugation (10 min at 13000 rpm) the combined MeOH extract was evaporated using Zymark Turbo Vap evaporator, USA, at 50 °C. The dried MeOH extract was further extracted with 1.5 mL and subsequently with 1 mL of 3 % HCl (twice) in an ultrasonic bath at 50°C for 30 min. After centrifugation (10 min at 13000 rpm), the combined aqueous extract was cooled at 4°C and treated with 25% ammonium hydroxide to reach pH 8-9. Alkaloids were extracted three times with $CHCl_3$ (4 mL each). Combined $CHCl_3$ extracts were dried (constant weight) and then dissolved in 1 mL of MeOH for HPLC analysis (Figure 3-7).

- *Quantification of indole alkaloids using HPLC:*

The separation was performed on an X select C_{18}-column, 2.5 µm, 100A, 4.6 x 100 mm from Waters, using a gradient system. Solvent A: acetonitrile; B: ammonium acetate (15 mM), containing 0.2% triethylamine, adjusted with formic acid to pH 3.5. 0 min: 15% A, 85% B; 5 min: 20% A, 80% B; 20 min: 30% A, 70% B; 23 min 60%A, 40% B; 24min: 80% A, 20% for 5 min. The flow-rate was 700 µL/min. The overall runtime was 35 min at a column temperature of 40°C. Alkaloids were quantified at 280 nm using a photodiode array (PDA) detector.

Quantification was based on the calibration curve using vincamine and vincadifformine standards (1 mg/mL), which were diluted down to 0.05 mg/mL by MeOH containing 300 mg/L strychnine as internal standard (IS). Thus, for IS, ratios of peak areas are used in quantification instead of peak area: a plot of the area ratios versus concentration ratios. The response factor (R) can be calculated from a calibration plot of A_X/A_{IS} versus C_X/C_{IS}, where the response factor is the slope. Then, the unknown concentration can be calculated from the measured area ratio from the experiment.

$$C_x = \frac{A_x/A_{is}}{R} \times C_{is}$$

(A: peak area; C: concentration; R: response factor)

The standards of methoxyvincamine, minovincine and minovincinine were not available, hence quantification of these compounds was based upon the UV response factor of vincamine and

vincadifformine their putative precursors. Therefore, methoxyvincamine was calculated as vincamine; whereas minovincine and minovincinine were calculated as vincadifformine.

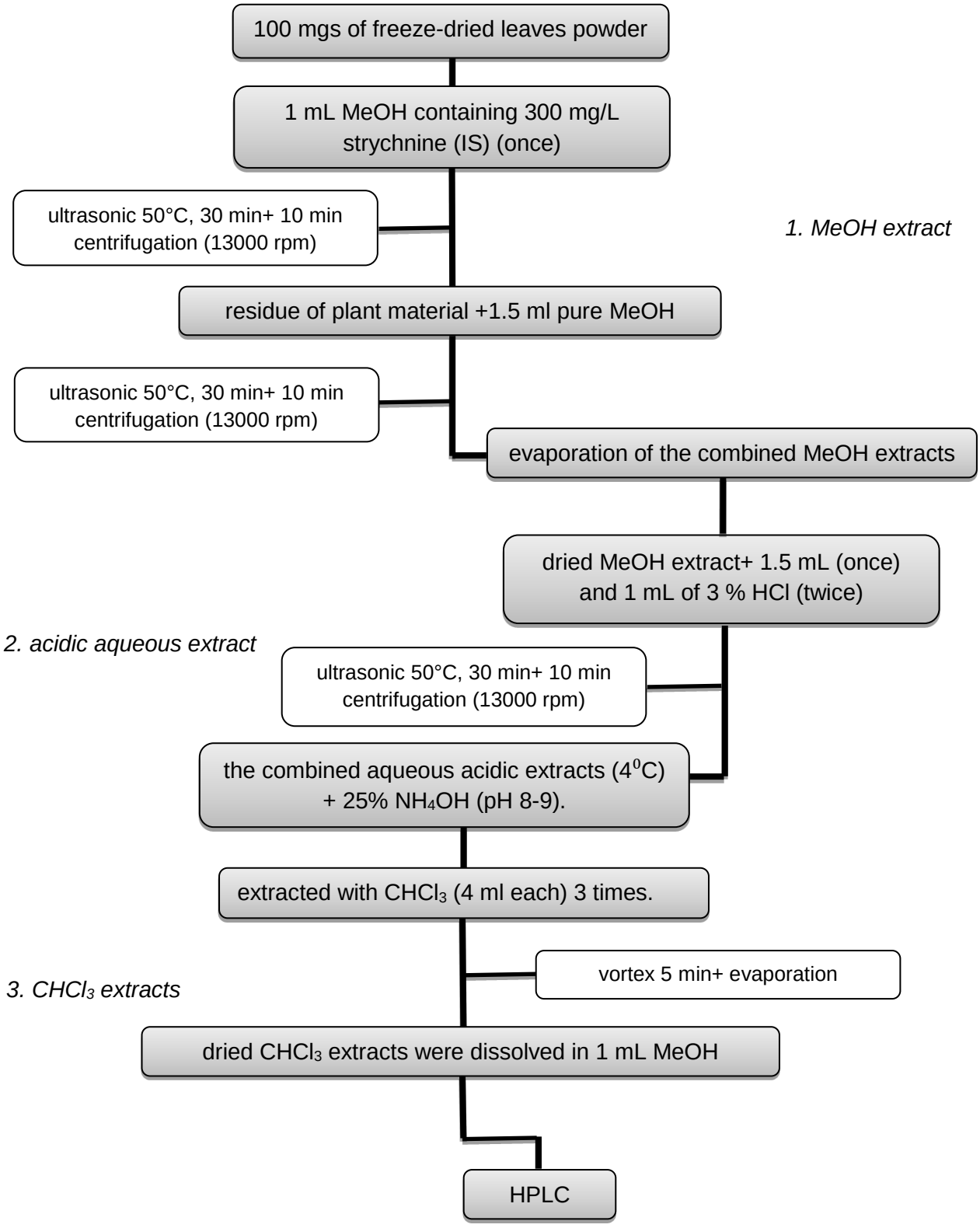


Figure 3- 7: The extraction procedure of indole alkaloids from *V. minor* leaves.

- *Data analysis and statistics:*

Because of unbalanced design, data from alkaloid concentration, of each period of time (1, 4, 8, 14 days) were analyzed by General Linear Model (GLM) and the significance of the treatment means was determined using the Least Significant Difference (LSD) test at $P \leq 0.05$. All analyses were performed using the SPSS statistical package release 24 (SPSS Inc., Chicago, IL, USA).

- *Quantification of indole alkaloids using LCMS* (*performed by Dr. Ulrike Beutling Chemical Biology, Helmholtz Centre for Infection Research, Braunschweig)*

The Total Ion Chromatogram (TIC) of the LC-MS runs could be used for quantification, assuming that the analyzed substances have similar ionization behavior. For the calculation of the peak areas, the Find-Molecular-Feature function of the Data Analysis Program (Bruker Daltonics) was used. Accordingly, further analyses of the MeJA-treated as well as of the control plants were conducted by using LC-MS analyses. For this, the old leaves were extracted as mentioned above. Strychnine was added as internal standard to the solvent used in the extraction. After analysis of the samples by LC-MS, the peak areas are calculated. Subsequently, peak areas are normalized to the internal standard strychnine. The separation was performed on a Kinetex C_{18}-column (1.7 µm, 100 Å, 150 × 2.1 mm) from Phenomenex, using a gradient system as mentioned above.

- Mass spectrum calculation was performed with Line Spectra
- Compounds - Molecular Features function: This function is performed using the Compounds - Molecular Features command. It finds compounds on an LC-MS chromatogram trace. It searches the selected chromatogram within the selected retention time range and generates compounds with Mass Lists for each compound. Compounds - Molecular Features is based on the principles that the traces of all ions belonging to one compound have a high time-correlation and that the chromatographic peaks of these ions are strongly overlapping. Furthermore, the algorithm interprets mass distances (e.g. as distances of isotopes or distances according to different charges) between ions. Finally, a compound is a collection of ions satisfying the two criteria for high correlation in time and the mass distances can be interpreted. The core algorithm is applied to line spectra. If no line spectra are available or an option to use profile spectra is set, then the line spectra are computed in a pre-processing step using the current method parameter settings for Mass List finding.

- The settings used for peak finding in Data Analysis Program:
  - *S/N* Threshold: 5
  - Correlations coefficient threshold: 0.7
  - Minimum compound length: 10 spectra
  - Smoothing width: 2

3.5 Effect of inhibitors on the MeJA and H_2O_2 induced conversion of indole alkaloids

- *Resveratrol application on the irrigated water*

100 mL resveratrol solution (200 mg/L) was added to the irrigation water, resulting in a dose of 20 mg resveratrol per pot. Triton X-100 (0.2%) was used for solubilization of resveratrol and applied to the soil as a negative control. The concentration of resveratrol used in this study reflected the concentration found in *Fallopia japonica* leachates and was based on the previous results of Fan et al. (2010), and Tucker Serniak, (2016). Solutions of resveratrol and Triton X were applied to the soil every five days and plants were harvested four times before next addition of the second treatment during the experiment. Six replicates were sampled at each harvest (48 pots).

- *Elicitor and inhibitors application as a spray*

Application of MeJA, H_2O_2, naproxen, and resveratrol were performed by using a 0.5 mM, 20 mM, 75 mg/L, and 100 mg/L solutions, respectively which contained Triton X (0.2%). Naproxen (75 mg/L) was applied directly after MeJA and H_2O_2 treatment. In the same manner, resveratrol (100 mg/L) and MeJA was applied concomitantly (Figure 3-8). For all approaches, plants were sprayed with 15 mL of the solutions. In the case of MeJA, treated plants were taken out of the growth area and transferred in an open area about 30 m distance from the control plants and sprayed with MeJA solution on both sides of leaves until liquid dripped from the leaves.

Three plants were collected before the application of the treatment. After three days of the first application of treatments, the first harvest was performed. The remaining plants were sprayed again and harvested at nine days from the beginning of the experiment. For each treatment (control, MeJA, H_2O_2, MeJA + naproxen, H_2O_2 + naproxen, and MeJA + resveratrol), three replicates were sampled at each harvest. Accordingly, in total 36 pots had been grown.

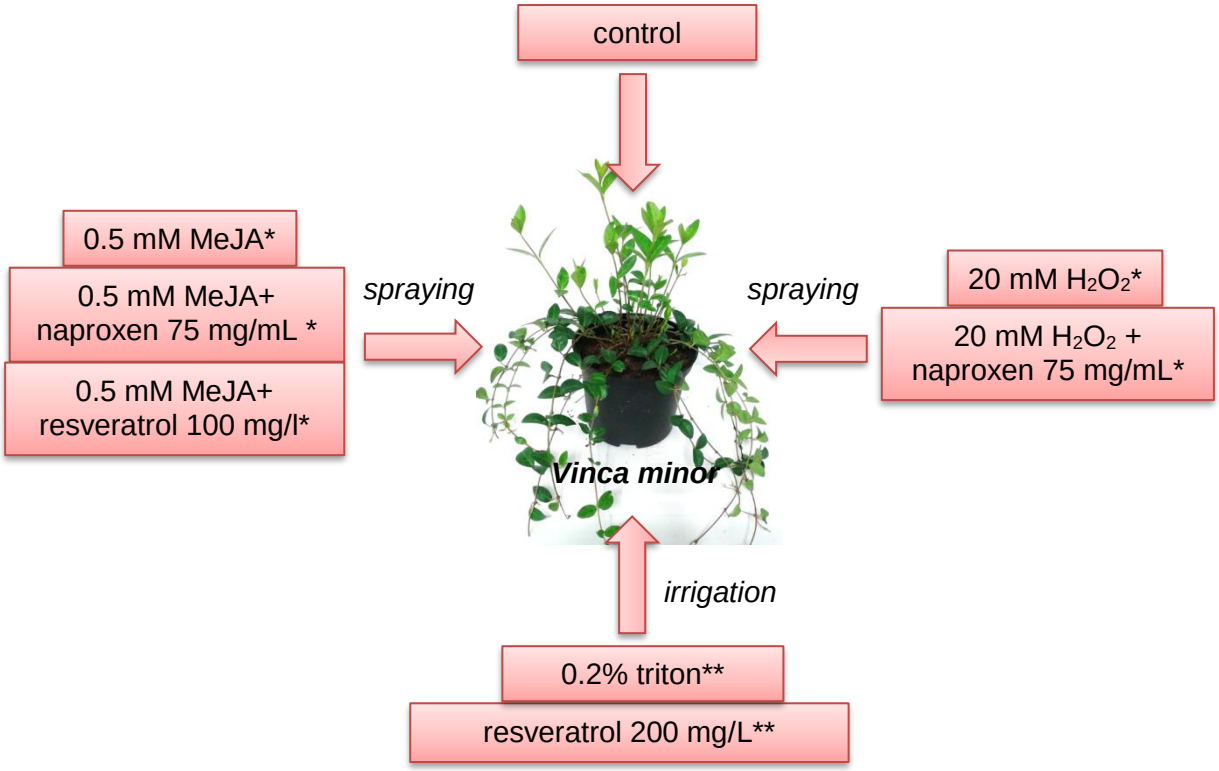


Figure 3- 8: Experimental design of the pot experiment with *V. minor*. *treatment was applied as spray 15 mL/plant, ** treatment was applied as irrigation in the soil 100 mL/pot.

3.6 Extraction of indole alkaloids from fruits and flowers of *V. minor*

Fruits and flowers were collected from mature *V. minor* plants in April 2017 (Figure 3-9). The extraction and analysis of indole alkaloid pattern were performed as mentioned above.

Figure 3- 9: Fruits and flowers separated from mature *V. minor* plant.

Chapter 4: Results

Nowadays, there is a tremendous demand for further alternative indole alkaloid-based pharmaceuticals derived from *Vinca minor* (Hasa *et al.*, 2013). It has been reported that *V. minor* contains a large amount of a wide spectrum of indole alkaloids. So far, more than 50 indole alkaloids have been extracted and isolated from lesser periwinkle (Table A-1). Although a quite different composition of indole alkaloids of *V. minor* is outlined in the literature, in all cases the major alkaloid in the leaves is reported to be vincamine (Proksa and Grossmann, 1991; D'Amelio Sr. *et al.*, 2012). Due to its pronounced cerebrovasodilatory and neuroprotective activity, many remedies containing vincamine are promoted to treat memory disorders or as stimulants for the CNS (Hasa *et al.*, 2013). In this sense, the major goal of this investigation is to elucidate, how the quality and composition of alkaloid extracts from *V. minor* could be altered. As known for alkaloids, the content of vincamine and other monoterpenoid indole alkaloids (MIAs) varies largely depending on growth, climatic conditions, the season, and putative stress situations (Boyadzhiev *et al.*, 2002). Accordingly, as outlined in the chapter "Scientific background", the application of growth regulators, being part of the various signal transduction chains, seems to be a promising approach to alter the alkaloid composition in plants.

In this study, mature *V. minor* (Apocynaceae) plants of defined physiological status were used as a model system to investigate the effect of elicitation with methyl jasmonate (MeJA) and other growth regulators on the composition and pattern of alkaloids with special emphasis on the appearance of novel, so far unknown indole alkaloids.

4.1 Optimization of extraction and HPLC analyses of MIAs from *V. minor*

As outlined above, quite different pattern of MIAs in *V. minor* has been reported so far. Accordingly, the first task was the sound investigation of the alkaloids present in the experimental plants used for this study. Based on the various protocols described so far, a sound and reliable methods for extraction, identification and quantification of the MIAs present in the leaves of *V. minor* had to be implemented.

4.1.1 Internal standard

Vincamine in plants was reported to be efficiently extracted with organic solvents or acidic aqueous solution or in combinations (Verma *et al.*, 2014b; J Liu *et al.*, 2015). One of the first steps was the selecting of an appropriate internal standard, which should be employed to minimize methodological errors and to consider the putative differences in the extraction

efficiency. In principle, the standard has to have three important properties. First, it must reveal a quite similar solubility in various solvents and thus a nearly identical extraction efficiency, secondly, there should be no interference in the HPLC analysis which the corresponding compounds present in the plants to be extracted; and finally, the internal standard has to be detectable by the same detection method as the molecules of interest, i.e., the indole alkaloids isolated from *V. minor*, which are registered by a photodiode array detector (PDA) (Hisiger and Jolicoeur, 2007). Strychnine turned out to be the best choice as the internal standard for this study.

4.1.2 Extraction method

In principle, the best extraction for alkaloids corresponds to an acidic aqueous extraction followed by alkalinization and transfer of the alkaloids into an organic solvent. Applying this classical procedure, an alkaloid solution is obtained, which is devoid of most compounds, which might interfere with the further analyses, e.g., chlorophylls, fatty acids etc. (Evans, Evans 2009). Yet, when applying this procedure, it turned out that the indole alkaloids from *V. minor* where not completely dissolved in the aqueous solution. In several papers, chloroform was used for the first extraction step of indole alkaloids (J Liu *et al.*, 2015). Unfortunately, also this method failed, too. Obviously, the polarity of the different alkaloids from *V. minor* is quite diverse, which hampers a complete extraction applying both of these methods.

Alternatively, the first extraction step was done using methanol. It turned out that the alkaloids are totally dissolved in the methanol. However, most of the undesired compounds were also present in the extracts. Thus, an alternative procedure had to be developed, which allows the complete extraction all various alkaloids from *V. minor*, while most of the undesired compounds remain in the solid phase. In this sense the following approach was developed: the finely ground plant powder was extracted with methanol (which contains strychnine as an internal standard). After centrifugation, the pellet was extracted twice with pure methanol. Subsequently, after evaporation, the residue of the methanolic solution was extracted three times with aqueous 3% HCl using an ultrasonic bath at 50°C for 30 min. Alternatively, also TFA (pH 1) could be used. To avoid decomposition of alkaloids, especially saponification of ester groups the combined acidic extracts were cooled at 4°C before alkalinisation by 25% ammonium hydroxide. Subsequently, indole alkaloids were extracted three times with chloroform. Combined chloroform extracts were dried (constant weight) and then dissolved in methanol for HPLC analysis. This procedure ensures that all alkaloids were extracted efficiently

and prevents the occurrence of most undesired compounds in the extracts. Accordingly, it was used for all analyses presented in the thesis.

4.1.3 HPLC analyses

Because of the medicinal importance of indole alkaloids, several HPLC methods have been established for their separation and quantification (Gerasimenko *et al.*, 2001; Hisiger and Jolicoeur, 2007; J Liu *et al.*, 2015). An effective separation can be achieved using a saline buffer with potassium phosphate or ammonium acetate as an aqueous phase, and methanol or acetonitrile as an organic phase. As ammonium acetate buffer is more volatile than phosphate buffer, it can be easily removed. Thus, in all cases when MS (mass spectroscopy) should be applied ammonium acetate is preferred (Hisiger and Jolicoeur, 2007). Other studies are suggesting the use of ion-pairing additives such as TEA or TFA, which are commonly used for masking non-derivatized silanol groups and enhance peaks shape and reducing peak tailing. In addition, mobile phase pH is an important parameter and acidic pH (pH 3–4) enables better peak shape for basic alkaloids. At low pH, most of silanol groups are undissociated and ion-exchange interactions are then limited, even if the alkaloids are protonated (McCalley, 2002; Heijden *et al.*, 2004). Weak variations in the mobile phase preparation can change the degree of protonation of the alkaloids, and thus influence the elution pattern and the reproducibility in the retention times. Based on these data comprehensive alterations, changes and optimizations including the usage of different reversed-phase columns had been performed.

Best separation was achieved by using X select C18-column and applying a gradient system containing TEA and ammonium acetate buffer at pH 3.5 as an aqueous phase and acetonitrile as an organic phase (for details see material and method section). At the start of the work, just the Nucleosil 100-5µ RP-18 column was available. Accordingly, the first row of experiments was employed by using this column. Later, when X select was C18-column was on hand, this column, which reveals a better resolution and a much shorter run time, was applied. The best detection of the various MIAs was achieved by using a PDA detector at three different channels (280, 254 and 330 nm).

4.2 Composition of MIAs of mature and healthy *V. minor* plants

4.2.1 HPLC analyses

Using the optimized methods described above, the alkaloids from old and young leaves from mature *V. minor* plants were analyzed by using HPLC technique (Figure 4-1).

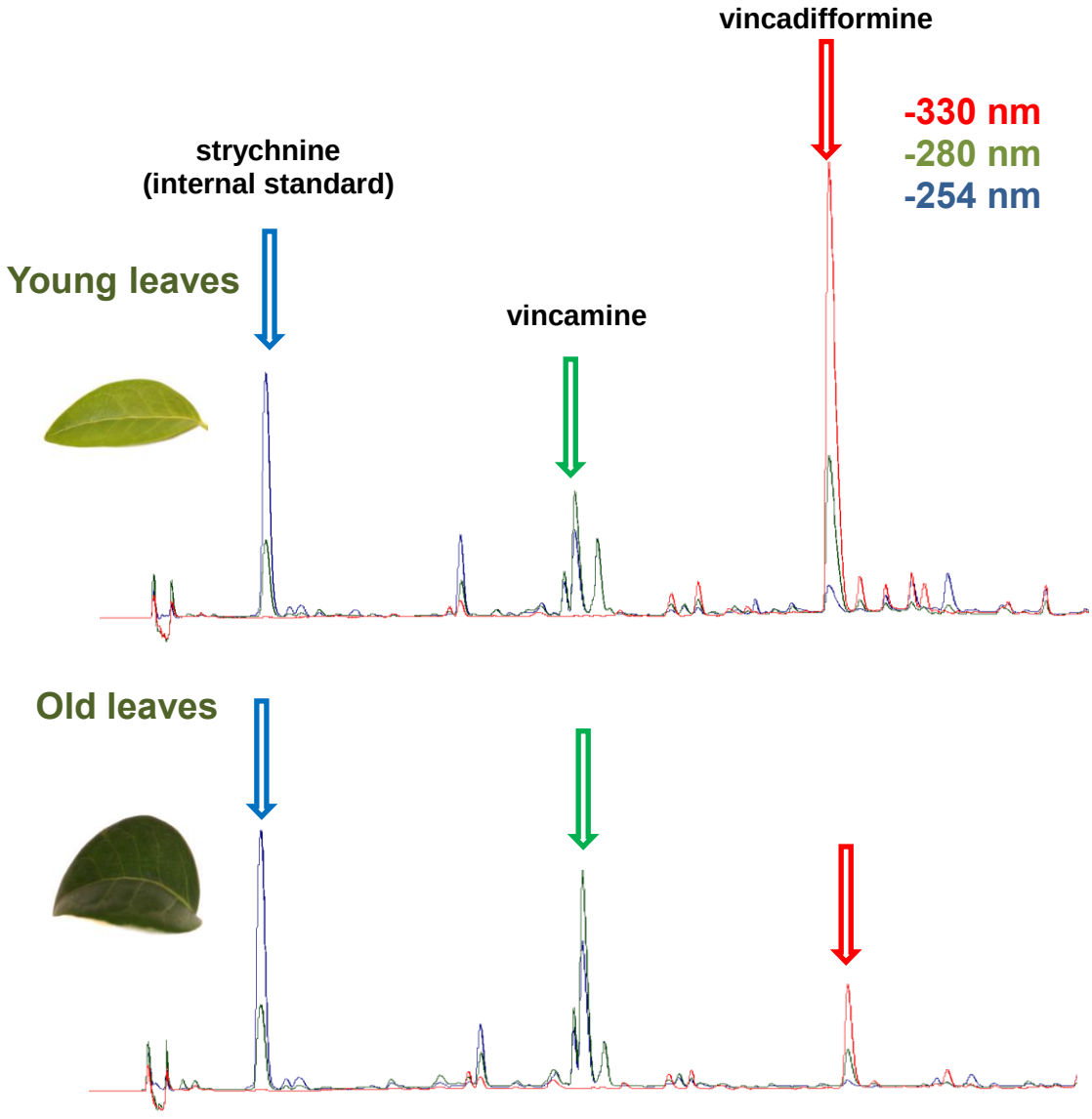


Figure 4- 1: HPLC-chromatogram of the alkaloids from young and old leaves of *V. minor* at different wavelengths using PDA detector.

It was clearly observed that vincamine represents the major alkaloid of old *V. minor* leaves (Figure 4-1). This could be easily deduced by comparing the retention time and UV spectrum of standard vincamine (Appendix: Figure A-1), with those obtained from the chromatogram of alkaloids in both young and old *V. minor* leaves (Figure 4-1). On the other side, the major alkaloid from young leaves was concluded to be vincadifformine. However, a reliable identification of this alkaloid required much more information and this cannot be achieved

before the isolation and spectroscopic identification by NMR techniques (see below for details). In the same manner, the identification of the other peaks displayed in Figure 4-1 also required much more information. Firstly, in an attempt to isolate these alkaloids, the extracts from the leaves (old and young) of *V. minor* were analyzed by ultra-performance liquid chromatography-mass spectrometry (UHPLC-MS). The related chromatogram, which revealed four main alkaloids' peaks (1, 2, 3, 4), is displayed in Figure 4-2. The corresponding data are given in Table 4-1.

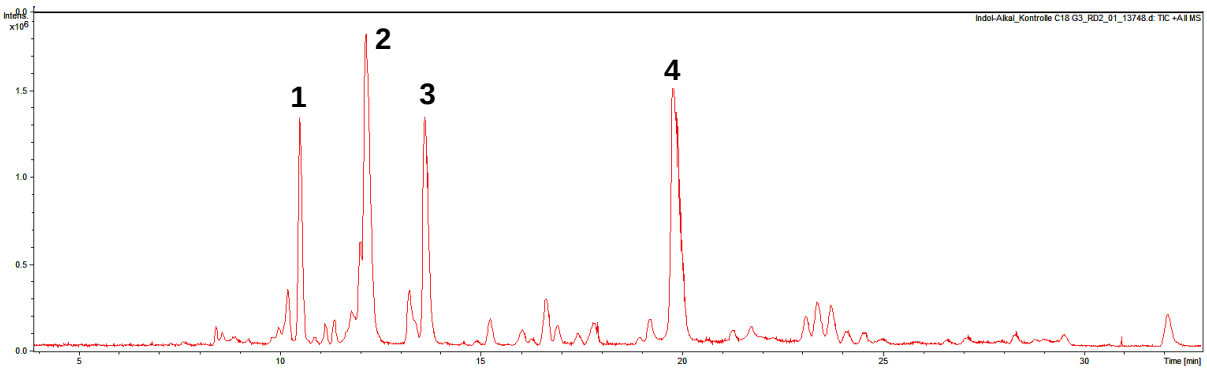


Figure 4- 2: LC-MS separation of the major indole alkaloids determined in *V. minor* control plants leaves.

Table 4- 1: UHPLC-PDA-MS data for MIAs of *V. minor* leaves in positive ion mode.

Compounds	t_R (min)	UV (λ_{max}) (nm)	MW	ESI^+ (*m/z*) (rel. int. %)	Molecular formulae $[M+H]^+$
1	10.49	208, 245, 312	354	355 (100)	$C_{21}H_{27}N_2O_3$
2- Vincamine	12.14	220, 270	354	355 (100), 337 (60)	$C_{21}H_{27}N_2O_3$
3	13.62	220, 270	322	323(100)	$C_{20}H_{23}N_2O_2$
4	19.77	221, 330	338	339 (100), 307 (83)	$C_{21}H_{27}N_2O_2$

Although the molecular masses were determined – apart from vincamine - no reliable assignment of the different peaks was possible on the basis of the properties mentioned in Table 4-1. In consequence, the relevant compounds had to be isolated in order to elucidate their structures by applying ^1H, ^{13}C NMR, COSY, TOCSY, ROESY, HSQC, and HMBC experiments and high-resolution mass spectrometry (HR-MS).

4.2.2 Identification and characterization of the alkaloids from mature *V. minor* leaves (control plants)

For identification of the major peaks in *V. minor* leaves, the alkaloidal extract was subjected to Nucleosil 100-5µ RP-18 semi-preparative column using a gradient system (for details see material and method). The isolated structures of vincamine together with three highly purified compounds were characterized by ^{1}H, ^{13}C NMR, COSY, TOCSY, ROESY, HSQC, and HMBC experiments and further confirmed by high-resolution mass spectrometry (HR-ESI-MS).

Compound 1:

Compound **1** was obtained as an amorphous powder. The molecular formula of **1** was determined as $C_{21}H_{26}N_2O_3$ on the basis of its HR-ESI-MS at *m/z* 355.2025 $[M+H]^+$, (Calculated *m/z* for M+H, 355.2022). The MS^2 spectra of compound **1** showed a peak at *m/z* 252, resulting from loss of both the ester group and a C_2H_5N fragment (Appendix, Figure A-2). In addition, a characteristic ion of a hydroxy indole moiety at *m/z* 160.0756 (143 Da + OH) might be generated by the loss of a terpene moiety by Retro Diels Alder (RDA) cleavage of the C-ring (Figure 4-4; (Kumar *et al.*, 2016a)). The UV spectrum of **1** (MeOH) exhibited maxima at λ_{max} 208 and 312 nm (Figure A-2).

The corresponding NMR spectra are displayed in the appendix (Figure A-3: A-9). The relevant NMR-data extracted from these figures are presented in Table 4-2.

The ^{1}H NMR spectrum of **1** (Table 4-2 & Figure A-3) showed three ABX-system protons at δ_H 6.33 (1H, d *J*= 1.8 Hz, H-9), 6.42 (1H, dd *J*= 7.8, 1.8 Hz, H-11) and 6.47 (1H, d *J*= 7.8 Hz, H-12) assigned for a 10-substituted dihydroindole (Balsevich *et al.*, 1982). Two downfield methyl singlets at δ_H 3.72 and 2.53 were assigned to the ester's methyl and the *N*-methyl groups, respectively (Balsevich *et al.*, 1982). An olefinic proton at δ_H 5.34 (1H, q, *J*= 7.10 Hz, H-19) and a methyl signal at δ_H 1.52 (3H, dd, *J* = 7.0, 1.72 Hz, H-18) established the presence of an ethylidene group (Balsevich *et al.*, 1982).

Accordingly, the ^{13}C NMR spectrum of **1** (Table 4-2 & Figure A-4) displayed characteristic signals for an oxygenated aromatic tertiary carbon at δc 150.7 (C-10), a nitrogenated aromatic tertiary carbon at δc 145.8 (C-13), a quaternary aromatic carbon at δc 141.5 (C-8), an aliphatic quaternary carbon at δc 42.4 (C-7), a pair of olefinic carbons at δ_C 139.5 (C-20) and 118.2 (C-19), an ester carbonyl carbon at δ_C 172.5 (C-17), three methyls at δ_C 35.3, 51.4, and 12.7 (C-N_1, C-22 and C-18) and four methylenes at δ_C 49.7, 30.8, 32.5, and 54.1 (C-5, C-6, C-14, and C-21) indicating a dihydroakuammiline skeleton (Balsevich *et al.*,

1982). However, the COSY and HMBC data (Table 4-2) also disclosed partial structures that are reminiscent of a dihydroakuammiline except that the $NCHCH_2CH$ partial structure, corresponding to C(3)-C(14)-C(15), has been extended to include CHCO fragment, corresponding to C(16)-C(17). The location of a methyl ester at C-16 was established from the HMBC correlation of the methine proton H-16 to the ester carbonyl C-17. The ethylidene group substituent, C(18)–C(19), attachment to C-20 was deduced by the HMBC correlations of H-18, H-21 with C-20 Figure 4-3.

The above spectral data of **1** was in agreement with the published data of 10-hydroxycathofoline (Figure 4-3) isolated from *Vinca major* and its glycoside isolated from the root of *Catharanthus roseus* (Balsevich *et al.*, 1982; Wang *et al.*, 2012) respectively. It is worth noting that this is the first report for the isolation of 10-hydroxycathofoline from *V. minor*.

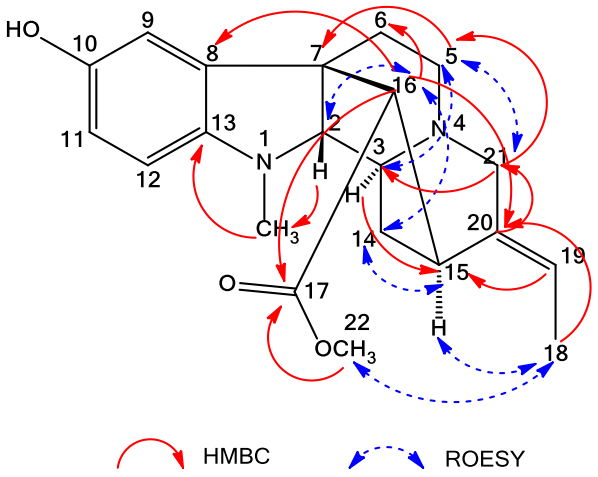


Figure 4- 3: Selected 2D NMR correlations for 10-Hydroxycathofoline (1).

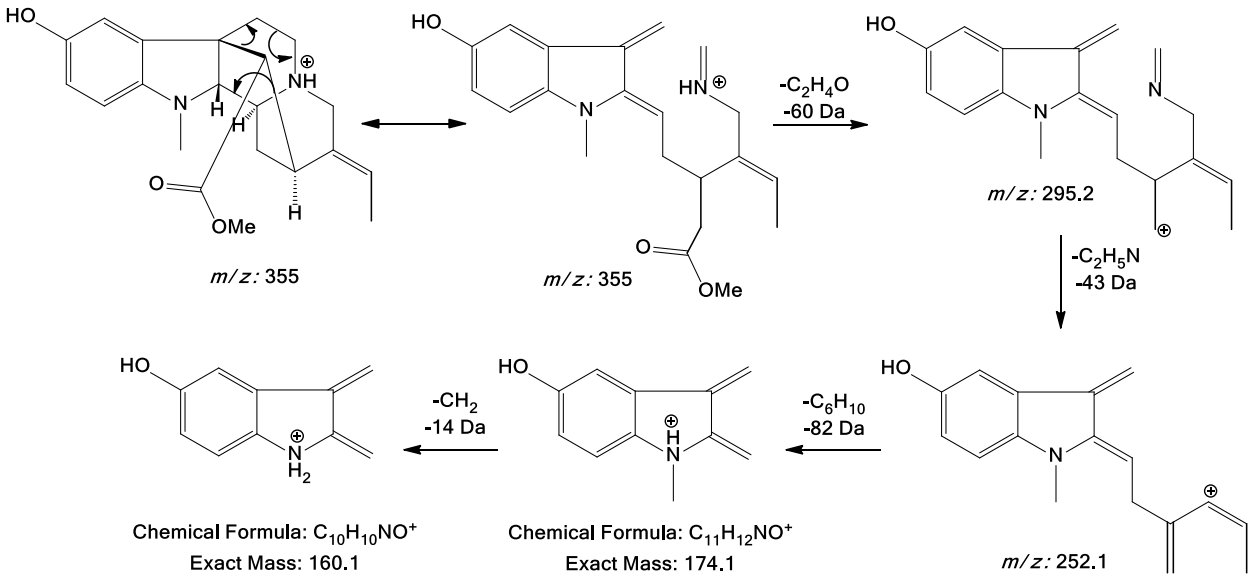


Figure 4- 4: Proposed mass fragmentation of 10-hydroxycathofoline (1) according to MS-MS data (Appendix: Figure A-2).

Table 4- 2: ^{1}H and ^{13}C NMR spectral data (700 MHz for ^{1}H, 175 MHz for ^{13}C) of 10-hydroxycathofoline (**1**) formic acid salt in DMSO-d_6.

Pos.	C Shift	XH_n	H Shift	H Multiplicity (*J* Hz)	COSY	TOCSY	ROESY	C HMBC
1Me	35.3	CH_3	2.53	m		6		2, 13
2	78.7	CH	2.42	m			14, 16	14, 1 Me, 16
3	46.9	CH	3.95	m	14	14, 14, 16, 15	5	7, 15
5	49.7	CH_2	2.53	m	6, 5	6, 6, 5	21, 6, 5, 3, 12	6, 7, 21
			3.63	m	6, 5, 6	6, 5, 6	6, 5	
6	30.8	CH_2	1.16	dd (15.2, 5.1)	6, 5	5, 6, 5	5	2, 5, 7
			2.97	m	6, 1Me, 5, 5	6, 5, 5	5	
7	42.4	C	---					
8	141.5	C	---					
9	108.8	CH	6.33	d (1.8)		12		7, 11, 13
10	150.7	C	---					
11	112.5	CH	6.42	dd (7.8, 1.8)	12			9, 10, 13
12	110.4	CH	6.47	d (7.8)	11	9	5	7, 8, 10
13	145.8	C	---					
14	32.5	CH_2	1.67	br dd (12.7, 2.8)	14, 15	14, 16, 15, 3	14, 2, 16	2, 3, 15, 16
			2.22	dt (11.3, 2.1, 2.1)	14, 15, 3	14, 16, 15, 3	14, 15, 21	
15	33.9	CH	3.45	m	14, 14, 16	18, 14, 14, 16, 3, 19	18, 14, 16	7
16	51.7	CH	2.95	m	15	14, 14, 15, 3	14, 2, 15	6, 8, 14, 17,20
17	172.5	C	---					
18	12.9	CH_3	1.42	dd (6.9, 1.7)	21, 19	21, 15, 21, 19	15, 22, 19	19, 20
19	118.2	CH	5.34	q (7.1)	18	18, 21, 15, 21	18, 21	18, 21, 15
20	139.5	C	---					
21	54.1	CH_2	2.90	br d (16.6)	21	18, 21, 19	5, 21, 19	5, 3, 20, 19, 15
			3.80	br d (17.4)	18, 21	18, 21, 19	14, 21, 22	
22	51.4	CH_3	3.72	s			18, 21	17

Note: Overlapped signals are reported without designating multiplicity.

Compound 2 (Vincamine):

Compound **2** was obtained as a solid white powder. The molecular formula of **2** was determined as $C_{21}H_{26}N_2O_3$ on the basis of its HR-ESI-MS peak at *m/z* 355.2024 $[M+H]^+$, (Calculated *m/z* for M+H, 355.2022) (Figure A-10). The MS^2 fragmentation of **2** (Figure A-10) showed the loss of a water molecule to give a peak at *m/z* 337 $[M - H_2O+H]^+$. In addition, a characteristic ion of indole moiety at *m/z* 144.0811 was generated by the loss of the terpene moiety through C-ring cleavage via Retro Diels Alder (RDA) followed by bond breaking between N_1 and C_{14} (Figure 4-6; Akhgari *et al.*, 2015; Kumar *et al.*, 2016a). The UV spectrum (MeOH) exhibited maxima at wavelengths 220 and 269 nm (Figure A-10).

The corresponding NMR spectra are displayed in the appendix (Figure A-11: A-16). The relevant NMR-data extracted from these figures are presented in Table 4-3.

^{13}C NMR spectrum of **2** (Table 4-3& Figure A-12) showed the presence of a hemiaminal quaternary carbon (δ_C 81.4, C-14), four quaternary aromatic carbons including a two nitrogenated tertiary carbons δ_C 124.7 (C-2), 135.1 (C-13) and two quaternary carbons δ_C 104.8 (C-7), 127.3 (C-8), an ester carbonyl carbon (δ_C 172.3, C-22) and seven methylene carbons, of which two were nitrogenated.

^1H NMR and HSQC data of **2** (Table 4-3 & Figure A-11) showed significant signals characteristic for four aromatic protons of the indole system at δ_H 7.53 (1H, d, J= 8.0 Hz, H-9), 7.23 (1H, m, H-10), 7.26 (1H, m, H-11) and 7.15 (1H, m, H-12). ^1H NMR spectrum also showed a methoxy signal at δ_H 3.87 (H-23), assigned to the ester group at C-14 based on its HMBC correlation with C-22. Also, the presence of a hydroxyl group at C-14 was confirmed from the HMBC correlation of the methylene protons at H-15 to C-14. The presence of a nitrogenated methine carbon at δ_C 59.7 (C-3) was deduced from the HMBC correlation between the proton signal at δ_H 4.65 (H-3) and C-2. The ethyl side chain substituent, C(20)–C(21) represented by the signals at δ_H 2.38, 1.60 (H_2-20) and 0.97 (H_3-21), was assigned to C-16 as deduced from the long-range coupling between H-20 to H-3 in the H–H ROESY spectrum and from correlation of H-20 and 21 to C-16 in HMBC spectrum.

Comparison of the above results with the reported data (Farahanikia *et al.*, 2011) clearly verified that this compound is vincamine (Figure 4-5).

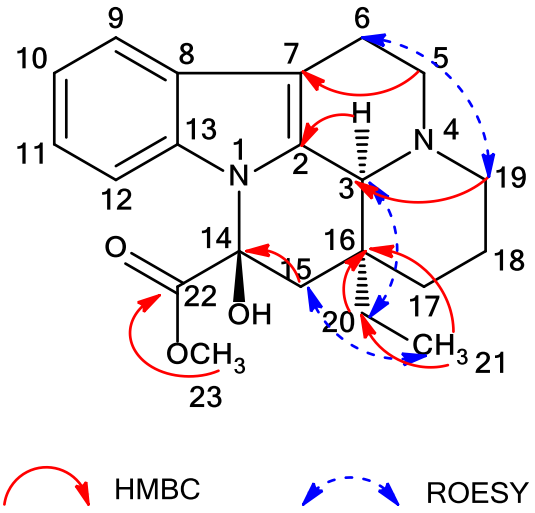


Figure 4- 5: Selected 2D NMR correlations for vincamine (2).

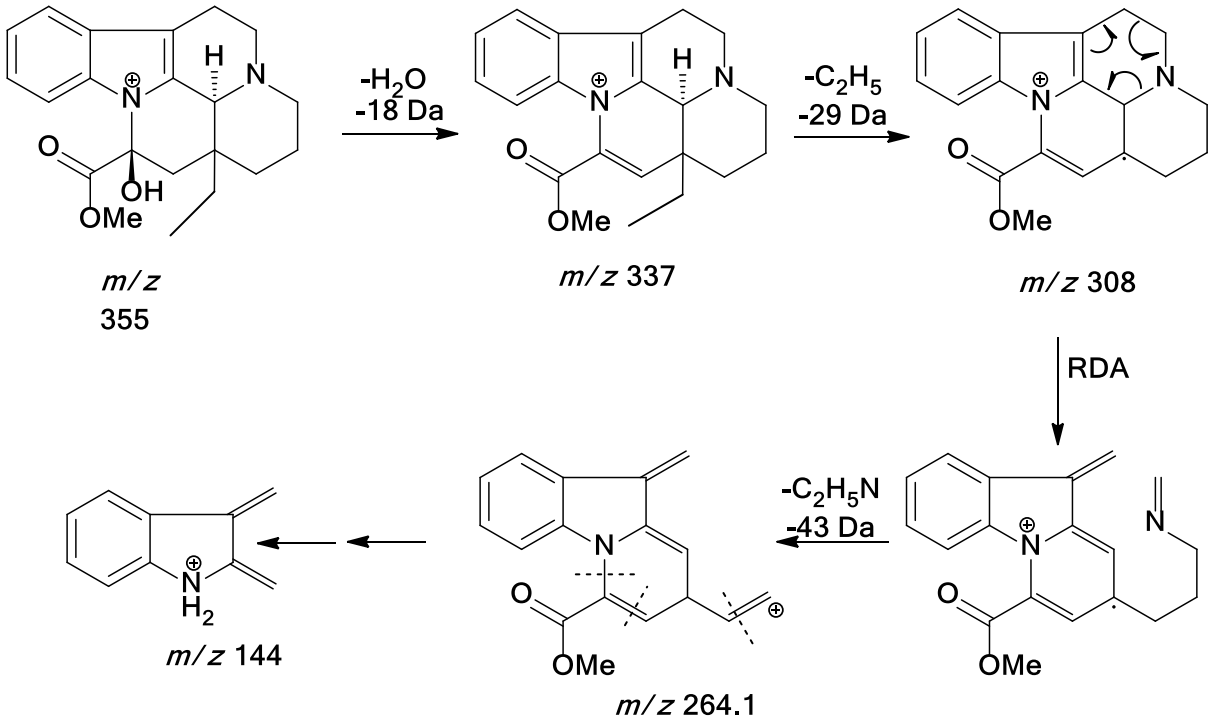


Figure 4- 6: Proposed fragmentation of vincamine (2) according to MS^2 data (Appendix: Figure A-11).

Table 4- 3: ^{1}H and ^{13}C NMR spectral data (700 MHz for ^{1}H, 175 MHz for ^{13}C) of vincamine (**2**) TFA salt in $CDCl_3$.

Pos.	C Shift	XH_n	H Shift	H Multiplicity (*J* Hz)	COSY	ROESY	C HMBC
2	124.7	C					
3	59.7	CH	4.65	br s		5, 15, 20	2, 7
5	50.9	CH_2	3.76	m	6	6, 18	7
6	16.1	CH_2	3.11	m	5	9, 5, 3	7, 2
7	104.8	C	---				
8	127.3	C	---				
9	119.0	CH	7.53	d (8.0)	10	10, 6	11, 7, 13
10	121.5	CH	7.23	m	9	9	8, 12
11	123.7	CH	7.26	m	12		9, 13
12	110.9	CH	7.15	m	11		10, 8
13	135.1	C	---				
14	81.4	C	---				
15	43.3	CH_2	2.28	d (14.7)			3, 13, 16, 14
			2.33	m	21, 20	21, 3	22, 3, 16, 17
16	35.8	C	---				
17	23.0	CH_2	1.85	m	17	17, 18	16, 18, 20
			1.68	br s	15	15	3, 19, 20
18	18.3	CH_2	2.12	m	18	18, 17	
			1.64	m	18	18	
19	44.5	CH_2	2.94	m	3	6, 3	
			3.35	br d (11.6)	3	3	17, 3
20	27.9	CH_2	1.60	m	21, 20, 15	20, 3	15, 21, 16, 17
	27.9	CH_2	2.38	m	21, 20	20	
21	7.3	CH_3	0.97	m	20, 15, 20	15	16, 20
22	173.3	C	---				
23	54.8	CH_3	3.87	m			22

Compound 3:

Compound **3** was obtained as a white powder. The HR-ESI-MS of **3** afforded the molecular ion peak at *m/z* 323.1757 $[M+H]^+$, suggesting the molecular formula $C_{20}H_{22}N_2O_2$ (Calculated *m/z* for M+H, 323.1760; Figure A-17). The MS^2 fragmentation of **3** (Figure A-17) showed the loss of methanol to give a peak at *m/z* 291$[M - CH_3OH + H]^+$, indicating the presence of methyl ester group. The UV absorption at 220 and 270 nm showed the presence of an indolenine chromophore, which was confirmed by the characteristic chemical shift of C-2 at δ_C 179.6 in the ^{13}C-NMR spectrum (Atta-ur-Rahman and Habib-ur-Rehman, 1986).

The corresponding NMR spectra are displayed in the appendix (Figure A-18: A-25). The relevant NMR-data extracted from these figures are presented in Table 4-4.

The ^{1}H NMR spectrum of **3** (Table 4-4, Figure A-18) showed four aromatic protons at δ_H 7.49 (br d J= 6.7 Hz), 7.29 (m), 7.44 (t J= 7.1 Hz) and 7.74 (br d J= 6.5 Hz) assigned to H-9, 10, 11, and 12, respectively, of the indole moiety, indicating the OH group at C-10 in **1** was absent in **3**. The methyl proton singlet at δ_H 3.77 (H-22) was assigned to the ester group at C-16. An olefinic proton at δ_H 5.85 (br q, J= 7.1 Hz) and a methyl signal at δ_H 1.67 (dd, J = 7.2, 2.3 Hz) indicated the presence of an ethylidene group. In addition, the signals observed at δ_H 5.28 (br s), 3.71(br s), and 3.67 (br d, J=16.4) corresponding to H (3), H (15), and H (21β), respectively, are characteristic of an akuammiline type alkaloid (Kan *et al.*, 1995).

^{13}C NMR spectrum of **3** (Table 4-4, Figure A-19) confirmed the presence of four downfield quaternary carbons including a nitrogenated tertiary carbons at δ_C 154.5 (C-13) and imine carbon δ_C 179.6 (C-2) of an indolenine nucleus. In addition, a quaternary olefinic carbon at δ_C 127.1 (C-20), and an ester carbonyl carbon at δ_C 170.5 (C-17) were also present.

The COSY and HMBC spectral data also disclosed partial structures that are reminiscent of an akuammiline skeleton, except that the NCHCH$_2$CH partial structure, corresponding to C(3)−C(14)−C(15), has been extended to include a CHCO fragment, corresponding to C(16)−C(17) (Lim *et al.*, 2007). The location of a methyl ester at C-16 was confirmed from the HMBC correlation of H-16 to C-17. The ethylidene group substituent, C(18)–C(19), attachment to C-20 was deduced from HMBC correlations of H-18 and H-19 with C-20. In addition, the assignment was confirmed by COSY and TOCSY experiments (Figure A-22, 23), which established the vicinal relationship between C-16H/C-15H and the allylic relationship between C-15H/C-18 protons.

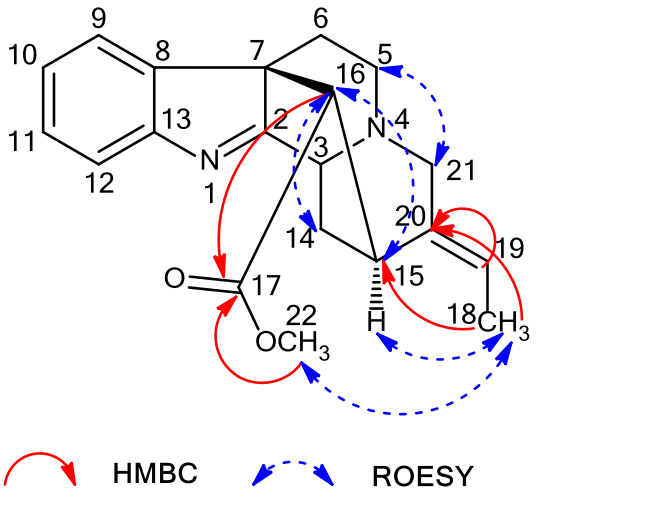


Figure 4- 7: Selected 2D NMR correlations for strictamine (3).

Based on the above spectral data and by comparison with literature data (Atta-ur-Rahman and Habib-ur-Rehman, 1986), compound **3** was confirmed as strictamine (Figure 4-7), which has been isolated before from *V. minor* (Mokrý *et al.*, 1967).

Table 4- 4: ^{1}H and ^{13}C NMR spectral data (700 MHz for ^{1}H, 175 MHz for ^{13}C) of strictamine (**3**) TFA Salt in $CDCl_3$.

Pos.	C Shift	XHn	H Shift	H Multiplicity (*J* Hz)	COSY	ROESY	C HMBC
2	179.6	C	---				
3	55.4	CH	5.28	br s	14		2, 15
5	51.3	CH_2	3.29	dd (13.8, 5.6)	5, 6	6, 5, 21, 6	21, 3, 6
			2.92	m	6, 5, 6	6, 5, 21	6
6	25.6	CH_2	3.82	br m	6, 5, 5	6, 5	5, 2, 16
			2.23	dd (15.9, 5.2)	5, 6	5, 5, 6, 9	5, 16, 8
7		C*	---				
8	144.4	C	---				
9	123.6	CH	7.49	br d (6.7)	10	22, 6	11, 13
10	127.4	CH	7.29	m	11, 9	11	8, 12
11	129.3	CH	7.44	t (7.1)	10, 12	10	9, 13
12	122.3	CH	7.74	br d (6.5)	11		8, 10
13	154.5	C	---				
14	33.6	CH_2	2.90	m	14, 3	14, 15, 21	21, 16, 17, 2, 3
			2.04	dd (14.8, 3.0)	14, 15	14, 15, 16	15, 2, 20, 16
15	31.0	CH	3.71	br s	14, 16	18, 14, 16, 14	14
16	54.1	CH	2.19	m	15	14, 15	2, 6, 20, 14, 15, 17
17	170.5	C	---				
18	13.3	CH_3	1.67	dd (7.2, 2.3)	21, 19	15, 22, 19	15, 19, 21, 20
19	127.2	CH	5.85	br q (7.1)	18	18, 21, 21	15, 20, 18, 21
20	127.1	C	---				
21	52.6	CH_2	4.48	br d (16.1)	18, 21	14, 21, 19, 5	19, 5
			3.67	br d (16.4)	21	5, 21, 19	19, 14, 3, 15, 5
22	52.2	CH_3	3.77	s		9, 18	17

*unidentified

Compound 4:

Compound **4** was obtained as a yellowish white powder. The molecular formula of **4** was determined as $C_{21}H_{26}N_2O_2$ on the basis of its HR-MS peak at *m/z* 339.2072 $[M+H]^+$, (Calculated *m/z* for M+H, 339.2073). The MS^2 fragmentations of **4** were predominated by the loss of methanol molecule to give *m/z* 307.1811 $[M - CH_4O + H]^+$ indicating the presence of

methyl ester group. The UV spectrum (MeOH) of **4** exhibited maxima at wavelengths 221, 293 and 330 nm (Figure A-26), which characteristic of aspidosperma-type alkaloids (Akhgari *et al.*, 2015).

The corresponding NMR spectra are displayed in the appendix (Figure A-27: A-33). The relevant NMR-data extracted from these figures are presented in Table 4-5.

^{1}H NMR and HSQC data of **4** (Table 4-5 & Figure A-27, A-32) showed significant signals for four A_2B_2-system protons at δ_H 7.84 (br d, *J* =7.5 Hz, H-9), 7.01 (t, *J*= 7.4, H-10), 7.21 (t, *J*= 7.6, H-11) and 6.85 (d, *J*= 7.5 H-12), of the indole moiety, and a NH signal at δ_H 9.01. It also showed a methoxy proton signal at δ_H 3.87 (H-23) assigned to an ester group.

^{13}C NMR spectrum of **4** (Table 4-5 & Figure A-28) showed the presence of seven methylene carbons including two nitrogenated methylene (δ_C 47.7, C-3 and 52.9, C-5) and a nitrogenated methine (δ_C 68.9, C-21). This was confirmed from the HMBC correlations between H-21 and C-5, 17 and 19. ^{13}C NMR spectrum further indicated the presence of a methoxycarbonyl (C-22), four quaternary carbons (C-7, C-8, C-16, C-20), and two nitrogenated (C-2, C-13) tertiary carbons. The location of a methyl ester was confirmed from ^{13}C NMR spectrum and from the HMBC correlation of the methyl proton signal, H-23 to C-16. The ethyl side chain substituent C(18)–C(19), represented by the signals at δ_H 0.94, 1.15 (H_2-19) and 0.64 (H_3-18) was deduced by the correlation between H-18 and 19 to C-20 (δ_C 35.1) in HMBC spectrum.

Comparison of the above results and the literature data (Wang *et al.*, 2017) confirmed that compound **4** is vincadifformine (Figure 4-8), which has been isolated before from *V. minor* (Mokrý *et al.*, 1967).

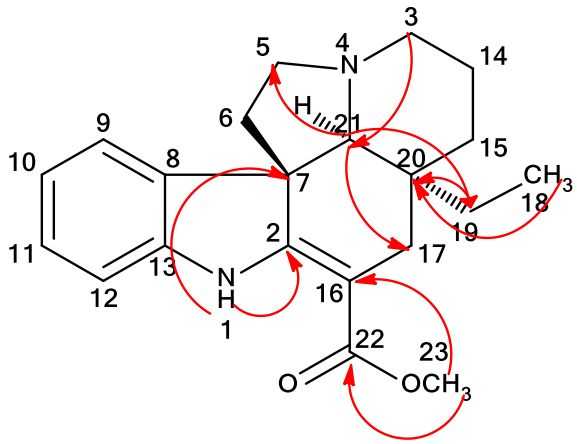


Figure 4- 8: Selected HMBC correlations for vincadifformie (4).

Table 4- 5: ^{1}H and ^{13}C NMR spectral data (700 MHz for ^{1}H, 175 MHz for ^{13}C) of vincadifformine (**4**) TFA Salt in $CDCl_3$.

Pos.	C Shift	XHn	H Shift	H Multiplicity (*J* Hz)	COSY	ROESY	C HMBC
2	163.6	C	---				
3	47.7	CH_2	3.57	br d (11.8)	14, 14, 3	3	5
			3.47	br d (12.5)	14, 3	3	21, 15
5	52.9	CH_2	3.75	br s	6, 5	6, 5	
6	42.8	CH_2	2.53	m	5, 5	5	8, 7
7	56.0	C	---				
8	133.5	C	---				
9	123.1	CH	7.84	br d (7.5)	10	21, 10	11, 7, 13
10	122.6	CH	7.01	t (7.4)	11, 9	11, 9	12, 8
11	129.1	CH	7.21	t (7.6)	12, 10	12, 10	13, 9
12	109.5	CH	6.85	d (7.5)	11	11, 1	10, 8
13	142.2	C	---				
14	14.7	CH_2	1.99	br d (13.1)	15, 14, 15, 3, 3	17	
			1.90	br d (14.2)	15, 14, 15, 3		
15	31.0	CH_2	2.06	br d (14.0)	15, 14, 14	18, 15	3, 21, 20, 17
			1.61	m	14, 14, 15	19, 15	20, 17, 14
16	90.0	C					
17	28.6	CH_2	2.44	d (16.6)	17	5, 14	22, 2, 19, 20, 16, 21
			2.56	m	17	18	19, 2, 20, 16, 22
18	6.4	CH_3	0.64	t (7.4)	19, 19	15, 17	19, 20
19	32.2	CH_2	0.94	br dd (14.2, 7.1)	18, 19	19, 15, 21	20, 15, 21, 18, 17
			1.15	dd (14.2,7.5)	18, 19	19, 21	18, 21, 20
20	35.1	C	---				
21	68.9	CH	3.97	m		19, 19, 9	6, 8, 7, 5, 19, 17, 20
22	168.2	C	---				
23	51.5	CH_3	3.81			17	22, 16
1		NH	9.01	s		12	7, 8, 2, 13

Summing-up – 4.2 Composition of MIAs of mature and healthy V. minor leaves

The mature and healthy leaves of the *Vinca minor* plants used in this study contain the four main alkaloids, i.e., vincamine, vincadifformine, 10-hydroxycathofoline, and strictamine, belonging to the three major groups of indole alkaloid:

1. Eburnamine group (vincamine)
2. Aspidospermine alkaloids (vincadifformine)
3. Akuammiline group (10-hydroxycathofoline, strictamine)

Whereas the occurrence of vincamine, strictamine and vincadifformine in *V. minor* was already reported earlier (D'Amelio Sr. *et al.*, 2012), 10-hydroxycathofoline, which is known to occur in *Vinca major* (Balsevich *et al.*, 1982) was isolated for the first time from *V. minor*. It is important to mention that the ^{1}H and ^{13}C chemical shifts deviated significantly from literature data, probably due to the protonation of the basic nitrogen atoms by TFA or protonation of formic acid during HPLC fractionation. This illustrates that metabolite de-replication using carbon chemical shifts is hampered in cases where the compounds act as weak acids or bases.

4.3 The effect of MeJA on the pattern of indole alkaloids of *V. minor*

4.3.1 Application of MeJA alters the alkaloid pattern in *V. minor* leaves (stressed plants)

In order to generate a massive stress situation, *V. minor* plants were treated two times with methyl jasmonate (MeJA) as described in the material and method section. After nine days, leaves had been harvested and the MIAs had been extracted and analyzed using the optimized method mentioned above

Surprisingly, the alkaloid pattern of *V. minor* leaves treated with MeJA was entirely different (Figure 4-9). Whereas the concentrations of strictamine (**3**) and 10-hydroxycathofoline (**1**) were not altered significantly, vincamine (**2**) and vincadifformine (**4**) contents were strongly reduced in the response to the MeJA treatment. Moreover, the concentration of several other indole alkaloids was increased massively.

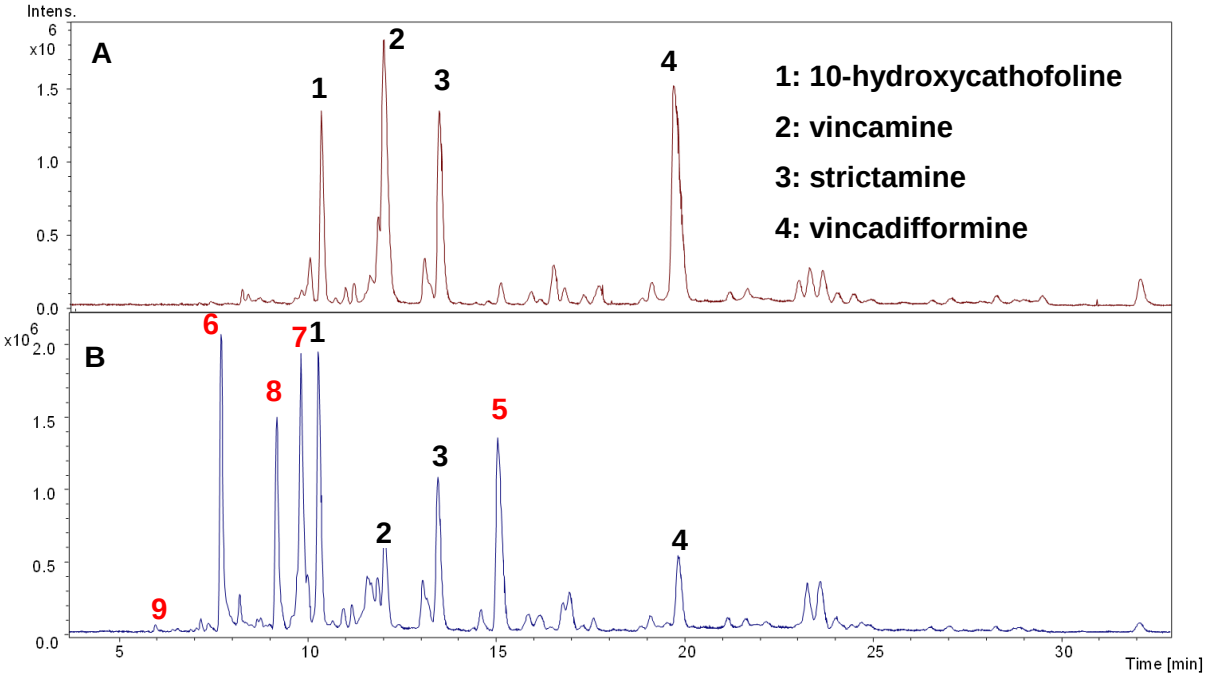


Figure 4- 9: LC-MS separation of the major indole alkaloids determined in *Vinca minor*. In the upper part (A) the composition of indole alkaloids extracted from control plants is shown, which already had been displayed in Figure 4-2, whereas in the lower part (B) the composition of indole alkaloids of *V. minor* plants nine days after treatment with MeJA are displayed.

In the same manner, as described for the four main alkaloids in the control plants (see chapter 4.2), the newly appearing compounds should be purified using semi-preparative HPLC and analyzed by HR-MS, ^{1}H, ^{13}C NMR, COSY, TOCSY, HSQC, and HMBC experiments. Unfortunately, it turned out that peak 8 consists of more than one alkaloid, which could not be separated using the preparative column. In consequence, **5, 6, 7** and **9** were purified and characterized. The analytical data of the various alkaloids of *V. minor* up-regulated by the MeJA treatment are compiled in Table 4-6.

Table 4- 6: UHPLC-PDA-MS data for MIAs of *V. minor* leaves treated with MeJA in positive ion mode.

Compound	**t_R (min)**	**UV (λ_{max}) (nm)**	**MW**	**ESI$^+$ (*m/z*) (rel. int. %)**	Molecular formulae $[M+H]^+$
5	15.17	224, 268	384	385 (100), 367	$C_{22}H_{29}N_2O_4$
6	7.87	220, 330	354	355 (100), 323 (59)	$C_{21}H_{27}N_2O_3$
7	9.99	221, 331	352	353 (100), 321	$C_{21}H_{25}N_2O_3$
8 *	9.35	240, 292	338	339 (100)	$C_{21}H_{27}N_2O_2$
9	5.9	219, 285	368	307, 210	$C_{21}H_{25}N_2O_4$

* In contrast to the preparative column, the separation using the ultra-performance HLPC allows separating compound **8** from the MIAs which interfered in the preparative separation. Accordingly, the mass of this alkaloid could be determined but no NMR analyses could be conducted.

4.3.2 Characterization and identification of the main alkaloids up-regulated in *V. minor* leaves after application of MeJA

The highly purified compounds (see Material and Methods, chapter 3, page 32) extracted from the MeJA-treated plants were characterized and structure elucidated using HR-MS and several NMR techniques (^{1}H, ^{13}C NMR, COSY, TOCSY, ROESY, HSQC, and HMBC experiments).

Compound 5:

Compound **5** was obtained as a colorless oil, $[\alpha]^{20}_{D}$ +117 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 224 NM (3.63) and 268 nm (3.06). The molecular formula of **5** was determined as $C_{22}H_{28}N_2O_4$ on the basis of its HR-ESI-MS peak at *m/z* 385.2130 $[M+H]^+$ (Calculated *m/z* for M+H, 385.2127). The MS^2 fragmentations of **5** were predominated by the loss of a water molecule to give a fragment at *m/z* 367.2022 $[M - H_2O+H]^+$ (Calculated *m/z* for $M - H_2O+H$, 367.2022) Figure (A-34).

The corresponding NMR spectra are displayed in the appendix (A-35: 41). The relevant NMR-data extracted from these Figures are presented in Table4-7.

^{1}H NMR and HSQC data (Table 4-7) showed the presence of three methyl groups (including two *O*-methyl singlets), seven methylene, a nitrogenated methine, and three aromatic methine protons.

The ^{13}C NMR spectrum additionally confirmed the presence of eight further carbons including a methoxycarbonyl, a hemiaminal carbon, three quaternary carbons, an oxygenated tertiary carbon and two nitrogenated tertiary carbons.

^{1}H and ^{13}C NMR data of **5** (Table 4-7) were closely related to those of vincamine (**2**), with the key difference in the replacement of the methine carbon (C-9) in **2** with an oxygenated tertiary carbon (δ_C 154.7) in **5**. Furthermore, an additional *O*-methyl signal (δ_H 3.89, δ_C 55.3) group was observed, which showed HMBC correlation to C-9. In addition, the splitting pattern of the aromatic protons H-10, H-11, and H-12 indicated that they are contiguous and *ortho* coupled as $J_{10,11} = J_{11,12} = 8.2$ Hz.

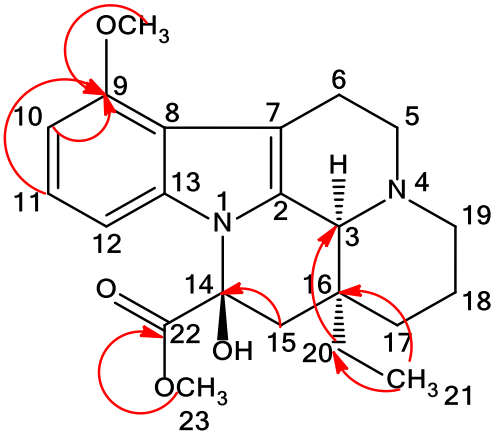


Figure 4- 10: Selected HMBC correlations for 9-methoxyvincamine (5).

Therefore, the structure of **5** was defined as 9-methoxyvincamine (Figure 4-10). A closely related compound, 11-methoxyvincamine, was reported earlier from *V. minor* (Štrouf and Trojánek, 1964; Szabó *et al.*, 1996), but 9-methoxyvincamine was isolated for the first time as a natural product.

Table 4- 7: ^{1}H and ^{13}C NMR spectral data (700 MHz for ^{1}H, 175 MHz for ^{13}C) of 9-methoxy-vincamine (**5**) TFA Salt in $CDCl_3$.

Pos.	C Shift	XHn	H Shift	H Multiplicity (*J* Hz)	COSY	NOESY	C HMBC
2	122.6	C	---				
3	60.0	CH	4.47	br s		5, 15, 20	2, 7, 16, 17, 19, 20
5	51.6	CH2	3.61, 3.72	dt (12.6, 6.2) m	5b, 6a, 6b 5a, 6a, 6b	3	19 7, 3
6	18.0	CH2	3.37 3.26	m m	6b 5a, 5b, 6a	6 6	2, 7, 8 2, 7
7	105.1	C	---				
8	117.6	C	---				
9	154.7	C	---				
10	101.5	CH	6.58	d (8.2)	11		8, 9, 12
11	124.8	CH	7.14	dd (8.2, 8.2)	10, 12		9, 13
12	103.8	CH	6.71	d (8.2)	11		8, 10
13	136.5	C	---				
14	81.4	C	---				
15	43.4	CH2	2.27	m		3, 21	3, 14, 16, 17
16	35.8	C	---				
17	22.9	CH2	1.85 1.66	td (14.2, 3.6) m	17 17	17, 17, 21	16, 20 3, 16, 19
18	18.2	CH2	1.62 2.18	m m	18 18	18 18	
19	44.5	CH2	2.94 3.30	m br d (10.8)	19b 19a	19b 19a	 3, 17
20	28.7	CH2	1.61 2.57	m dq (14.7, 7.4)	20, 21 20, 21	3, 20 20, 21	3, 15, 16, 17, 21 3, 15, 16, 21
21	7.4	CH3	0.98	t (7.4)	20, 20	15, 17, 20	20, 16
22	173.3	C	---				
23	54.8	CH3	3.87	s			22
9-OMe	55.3	CH3	3.90	s			9

Compound 6:

Compound **6** was obtained as a white powder. The molecular formula of **6** was determined as $C_{21}H_{26}N_2O_3$ on the basis of its HR-ESI-MS peak at *m/z* 355.2023 $[M+H]^+$ (Calculated *m/z* for M+H, 355.2022). The MS^2 fragmentations of **6** were predominated by the loss of a methanol molecule to give a peak at *m/z* 323.1760 $[M - CH_4O+H]^+$ (Calculated *m/z* for $M - CH_4O+H$, 323.1760), or a water molecule $[M - H_2O+H]^+$ to give a peak at *m/z* 337.1918 (Calculated *m/z* for $M - H_2O+H$, 337.1916). These fragmentations are indicating the presence of a methyl ester and a hydroxyl groups, respectively (Figure A-42). The UV spectrum (MeOH) exhibited maxima at wavelengths 220, 290 and 330 nm (Figure A-42), which characteristic of aspidosperma-type alkaloids (Akhgari *et al.*, 2015).

The NMR data of **6** (Table 4-8 and Figure A-43: 49) was closely related to those of vincadifformine (**4**), with the key difference in the presence of a 3H doublet at δ_H 0.73 (d, J = 6.5, H_3-18) coupled with a downfield quartet of one proton at δ_H 3.18(q, J = 6.5 Hz, H-19) suggested the presence of a hydroxyethyl group attached to C-20. In addition, the attachment of a hydroxyl group to C-19 was deduced by the downfield shift of C-19 (δ_C 64.9). This was confirmed from the HMBC correlations between H-19 and C-18, 17, 20, and 21. Thus, compound **6** was identified as minovincinine (Figure 4-11), which has been isolated before from *V. minor* (Plat *et al.*, 1962).

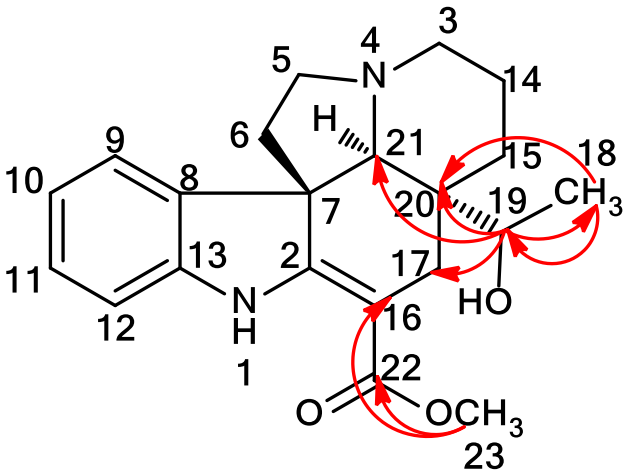


Figure 4- 11: Selected HMBC correlations for minovincinine (6).

Table 4- 8: ^{1}H and ^{13}C NMR spectral data (700 MHz for ^{1}H, 175 MHz for ^{13}C) of minovincinine (**6**) formic acid in DMSO-d_6.

Pos.	C Shift	XHn	H Shift	H Multiplicity (*J* Hz)	COSY	ROESY	C HMBC
2	166.5	C	---				
3	50.2	CH_2	3.05	m	3	14, 3, 5	14, 15, 21
			2.33	br d (3.0)	14, 3	14, 21, 3	21, 15
5	50.9	CH_2	2.44	m	6, 6, 5	6, 5, 9	7, 21, 6, 3
			2.84	m	6, 5	6, 5, 3	7, 6, 21
6	46.0	CH_2	1.48	m	6, 5	6, 5	8, 7
			1.85	br d (6.5)	6, 5, 5	5, 6	5, 8, 2, 7
7	55.0	C	---				
8	136.7	C	---				
9	120.4	CH	7.20	d (7.1)	10	5, 21	7, 11, 13
10	119.9	CH	6.79	t (7.4)	11, 9		12, 8
11	127.2	CH	7.07	t (7.8)	10, 12		13, 9
12	109.9	CH	7.01	d (7.5)	11	1	10, 8
13	143.8	C	---				
14	21.4	CH_2	1.53	m	14	14, 3, 21	
			1.72	m	14, 3	14, 17, 3	3, 15, 20
15	25.6	CH_2	1.50	m		17	20, 21, 3, 14, 17
			1.41	br d (4.3)		18, 21	3, 20, 17, 14, 19
16	91.3	C	---				
17	25.7	CH_2	2.56	m		15, 14, 19	20, 22, 16, 21, 19, 2
18	17.9	CH_3	0.73	d (6.5)	19	15, 21, 19	19, 20
19	64.9	CH	3.18	m	18	18, 21, 17	18, 17, 20, 21
20	42.1	C	---				
21	67.4	CH	2.55	m		18, 15, 3, 9, 14, 19	8, 3, 15, 7
22	167.7	C	---				
23	50.4	CH_3	3.64	m			16, 22
1		NH	9.45	s		12	7, 8, 2, 13

Compound 7:

Compound **7** was obtained as a white amorphous powder. The molecular formula of **7** was determined as $C_{21}H_{24}N_2O_3$ on the basis of its HR-ESI-MS peak at *m/z* 353.1869 $[M+H]^+$ (Calculated *m/z* for M+H, 353.1865). The MS^2 fragmentations of 7 were predominated by the loss of methanol molecule to give a peak at *m/z* 321.1604 $[M - CH_4O+H]^+$ (Calculated *m/z* for $M - CH_4O+H$, 321.1598) indicating the presence of a methyl ester group (Figure A-50). The UV spectrum (MeOH) exhibited maxima at wavelengths 221, 293 and 331 nm (Figure A-50), which characteristic of aspidosperma-type alkaloids (Akhgari *et al.*, 2015).

The NMR data of **7** (Table 4-9 and Figure A-51: 56) were closely related to those of vincadifformine (**2**), with the key difference in the replacement of the ethyl side chain with a methyl ketone as deduced by the δ_C 206.3 (C-19) and δ_H 2.14 (s, 3H, $COCH_3$). This was confirmed from the HMBC correlations between H-18, 17 and C-19. Compound **7** was assigned as minovincine (Laforteza *et al.*, 2013; Figure 4-12), which has been isolated before from *V. minor* (Plat *et al.*, 1962).

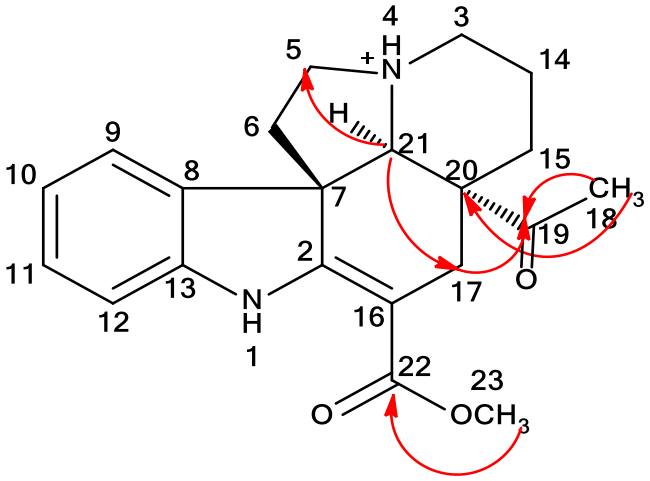


Figure 4- 12: Selected HMBC correlations for minovincine (7).

Table 4- 9: ^{1}H and ^{13}C NMR spectral data (700 MHz for ^{1}H, 175 MHz for ^{13}C) of minovincine (**7**) TFA salt in $CDCl_3$.

Pos.	C Shift	XHn	H Shift	H Multiplicity (*J* Hz)	COSY	ROESY	C HMBC
2	173.1	C	---				
3	46.8	CH_2	3.52	br s	14		
5	52.8	CH_2	3.87	m	6, 4NH+	6, 6, 17	21, 7
6	35.5	CH_2	2.97	m	6, 5	6, 9, 5	5, 2, 8
			2.83	br dd (14.3, 5.5)	6	6, 5	
7	60.2	C	---				
8	143.8	C	---				
9	123.7	CH	8.45	d (7.5)	10	6, 21, 11, 10	13, 11, 7
10	129.2	CH	7.45	t (7.5)	9	9	8
11	128.9	CH	7.40	m	12	9	13
12	122.2	CH	7.60	d (7.5)	11		10, 8
13	151.6	C	---				
14	14.3	CH_2	2.03	m	3		
15	29.8	CH_2	1.66	m	15	15, 21	17
			2.19	br d (13.6)	15	15, 17	3, 21
16	62.9	C	---				
17	43.8	CH_2	3.46	br d (16.4)	17	18, 15, 17	19, 22, 2, 21, 20, 16
			3.04	br d (16.6)	17	17, 5, 18	22, 16, 19, 15, 20
18	24.2	CH_3	2.14	s		23, 17	20, 19, 15
19	206.3	C	---				
20	48.1	C	---				
21	65.5	CH	4.47	m	4NH+	9, 15	7, 17, 5, 8
22	169.0	C	---				
23	54.4	CH_3	3.97	s		18	22
4NH+			13.49	br s			

Compound 9:

Compound **9** was obtained as colorless crystals. The molecular formula of **9** was determined as $C_{21}H_{24}N_2O_4$ on the basis of its HR-ESI-MS at *m/z* 369.1815 $[M+H]^+$ (Calculated *m/z* for M+H, 369.1814) (Figure A-57). The MS^2 fragmentation of **9** (Figure A-57) showed the loss of methanol to give a peak at *m/z* 337$[M - CH_3OH+H]^+$, indicating the presence of methyl ester group. The UV spectrum (MeOH) exhibited maxima at wavelengths 219 and 285 nm indicating the presence of an indolenine chromophore, which was confirmed by the characteristic chemical shift of C-2 at δC 186.9 in the ^{13}C-NMR spectrum(Atta-ur-Rahman and Habib-ur-Rehman, 1986; Figure A-57).

The NMR spectral data of **9** (Table 4-10 and Figure A-58:64) suggested an akuammiline skeleton, a closely related structure to that of **3** (strictamine).

^{1}H NMR spectrum of (**9**) showed three ABX-system protons at δ_H 6.96 (d *J*= 2.6 Hz, H-9), 6.65 (dd *J*= 8.3, 2.6, H-11), 7.27 (d *J*= 8.3, H-12) indicating the presence of a 10-substituted indolenine moiety. ^{13}C NMR spectrum (Table 4-10 and Figure A-59), confirmed the presence of two nitrogenated tertiary carbons (δ_C 147.9 & 186.9) and an oxygenated tertiary carbon (δ_C 155.4) confirming a 10-hydroxy substituted indolenine nucleus. In addition, substitution of the free hydrogen at H-16 in strictamine (**3**) with a CH_2OH group in **9** was also revealed. This was confirmed by the downfield shift of the carbon signals of C-7 and C-16 and from HMBC correlations of H-17 with these two carbons.

In conclusion, the above spectral data of **9** was in agreement with the published data of ercinamine (Figure 4-13), which has been isolated from *Vinca erecta* (Yagudaev *et al.*, 1983) and the tissue culture of *Catharanthus roseus* (Guéritte *et al.*, 1983). It is worth noting that this is the first report for the isolation of ercinamine from *V. minor*.

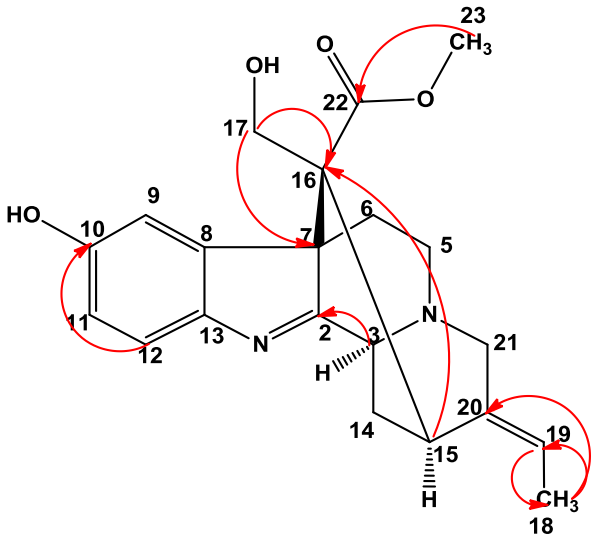


Figure 4- 13: Selected HMBC correlations for ercinamine (9).

Table 4- 10: Table 13. ^{1}H and ^{13}C NMR spectral data (700 MHz for ^{1}H, 175 MHz for ^{13}C) of ercinamine (**9**) formic acid salt in DMSO-d_6.

Pos.	C Shift	XHn	H Shift	H-Multiplicity (*J* Hz)	COSY	TOCSY	N/ROESY	C HMBC
2	186.9	C	---					
3	53.8	CH	4.28	d (4.5)	14, 14	14, 14, 15	14, 14	2, 7, 15, 21
5	51.3	CH_2	2.32	br d (4.5)	5, 6	6, 5, 17, 6	6, 5	3, 6, 7
			2.51	dt (3.6, 1.8)	5, 17	6, 5, 17, 6	6, 5	6
6	38.3	CH_2	3.42	m	6, 5	6, 5, 5	6, 17	6, 2, 7, 8, 16
			1.78	m	6	5, 5, 17, 6	5, 5, 6, 9	
7	58.2	C	---					
8	146.4	C	---					
9	112.5	CH	6.96	d (2.6)			6	7, 11, 12
10	155.4	C	---					
11	114.0	CH	6.65	dd (8.3, 2.6)			12	9, 13,
12	120.5	CH	7.27	d (8.3)			11	8, 10, 13
13	147.9	C	---					
14	30.2	CH_2	2.31	m	14, 3	14, 15, 3	14, 15, 21, 3	2, 15, 16, 20, 21
			1.73	m	14, 15, 3	14, 15, 3	14, 17, 17, 15, 3	
15	33.4	CH	3.62	br s	14	18, 14, 14, 21, 21, 3	18, 14, 14, 17	3, 7, 16, 20
16	59.7	C	---					
17	62.0	CH_2	2.77	d (11.8)	5, 17	5, 17	14, 17	3, 7, 15, 16, 22
			2.52	m	17	6, 5, 17	14, 17, 21, 6, 15	
18	13.13	CH_3	1.58	dd (7.2, 2.3)		21, 15, 21	15, 23, 19	19, 20
19	117.8	CH	5.37	br d (7.1)		21	18, 21	15, 18, 21
20	140.9	C	---					
21	53.3	CH_2	3.08	br d (17.2)	21	18, 15, 21, 19	17, 21, 19	5, 19, 20
			3.94	br d (17.2)	21	18, 21, 15	14, 21	
22	173.0	C	---					
23	51.4	CH_3	3.69	s			18	22
$CH_2O\underline{H}$			8.35	br s				

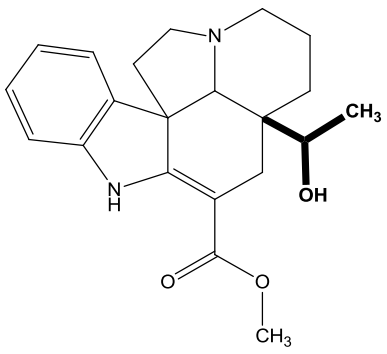
Compound 8:

In contrast to the other alkaloids mentioned above, the alkaloid denoted as compound **8** could not be isolated so far since the resolution of the semi-preparative column did not allow the isolation of this compound. Accordingly, only limited information on this alkaloid is available.

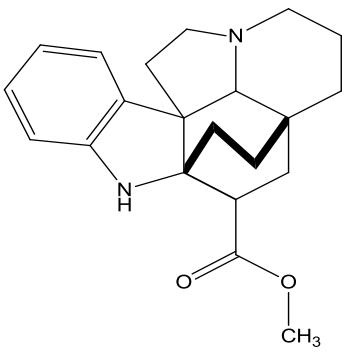
The molecular formula of **8** was determined as $C_{21}H_{26}N_2O_2$ on the basis of its HR-ESI-MS peak at *m/z* 339.2045 $[M+H]^+$ (Calculated *m/z* for M+H, 339.2073) (Figure 4-17). The UV (MeOH) spectrum exhibited a maximum at wavelength 240, 292 nm (Figure 4-14; Vachnadze *et al.*, 2010).

In comparison to the MS^2 fragmentations of minovincinine (**6)** and vincadifformine (**4)** which were predominated by the cleavage of the ester bond to give $[M - CH_4O + H]^+$ at *m/z* 323.1760 and 307.1811, respectively, compound **8** showed a peak at *m/z* 339.2045 $[M + H]^+$ as the base peak (Figure 4-17). The initial fragmentation pattern of compound **8** (Figure 4-15) involves fission of ring C, with expulsion of the ethylene group and the formation of an ion at *m/z* 311.18 $[M - 28+H]^+$, as in the aspidospermidine fragmentation (Saxton, 1983a) and followed by the loss of $C_2H_4O_2$ to give a peak at *m/z* 251.15. The MS^2 peak at *m/z* 251.15 of the previously mentioned compounds (**4**, **6**, and **8**) produced a series of fragment ions in MS^2 spectra. The driving force for this fragmentation is presumably the aromatization of the indole ring and the relief of ring strain in the fused hexacyclic system via Retro Diels Alder (RDA) cleavage of the C-ring (Saxton, 1983a). Further cleavage at the benzylic positions gives the piperidine-derived fragment at *m/z* 110.10, 96.08 and two aromatic ions at *m/z* 170.10 and 130.07 (Figure 4-16).

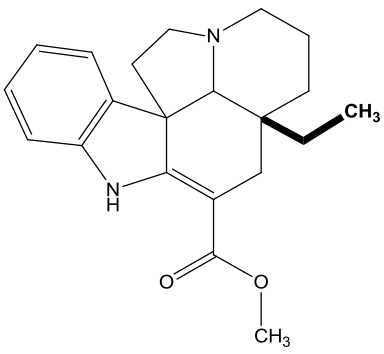
Therefore, the MS-MS spectrum of compound **8** was similar in fragmentation patterns to kopsinine determined by FAB-tandem mass spectrometry (Laprévote *et al.*, 1990).



Minovincinine (**7**)



Kopsinine (**8**)



Vincadifformine (**4**)

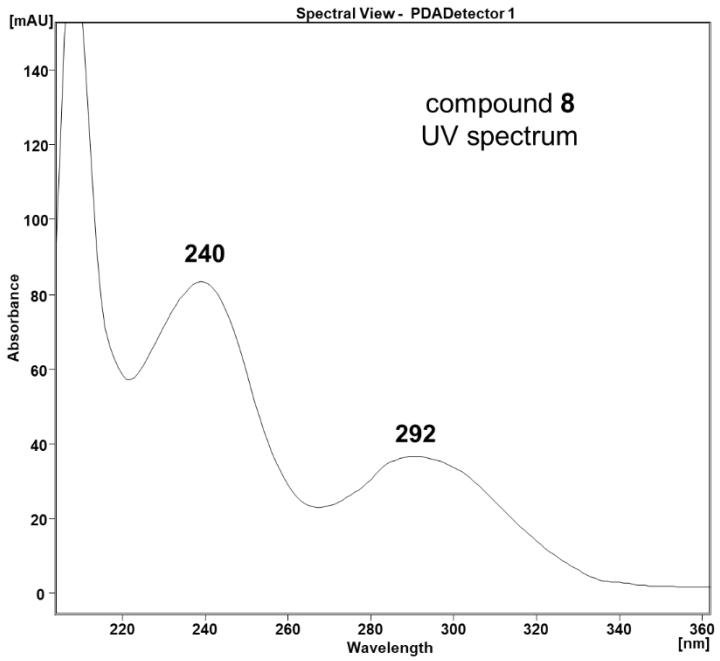


Figure 4- 14: UV spectrum of compound 8.

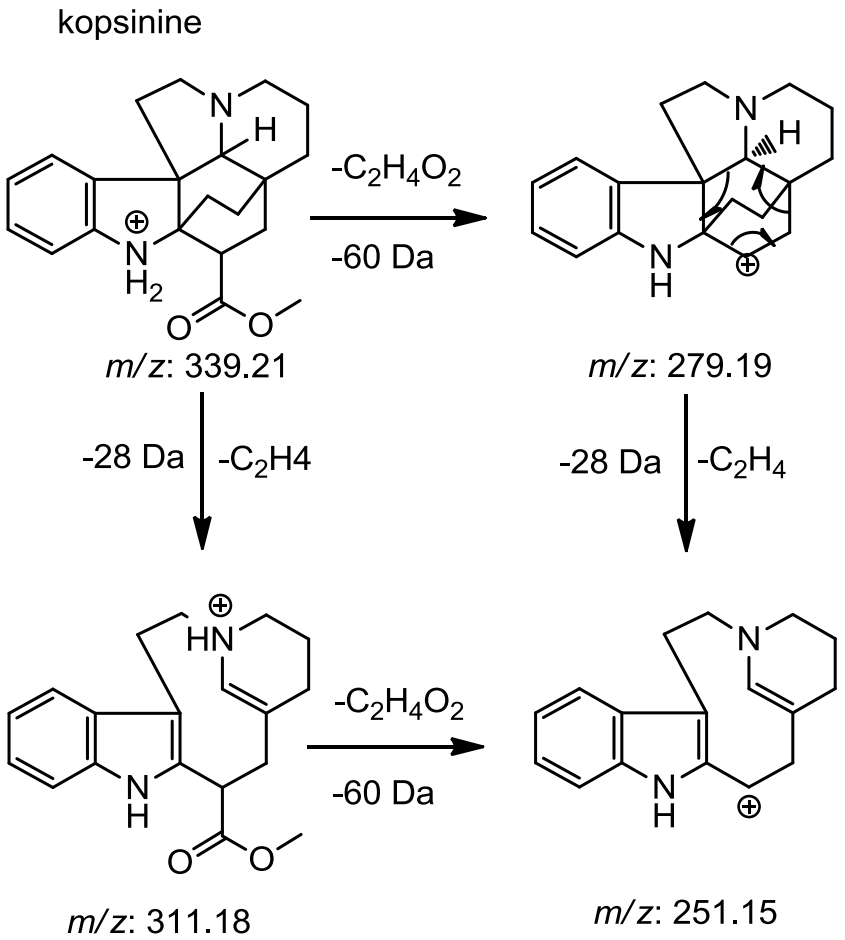


Figure 4- 15: Suggested scheme for fragmentation pattern of compound 8.

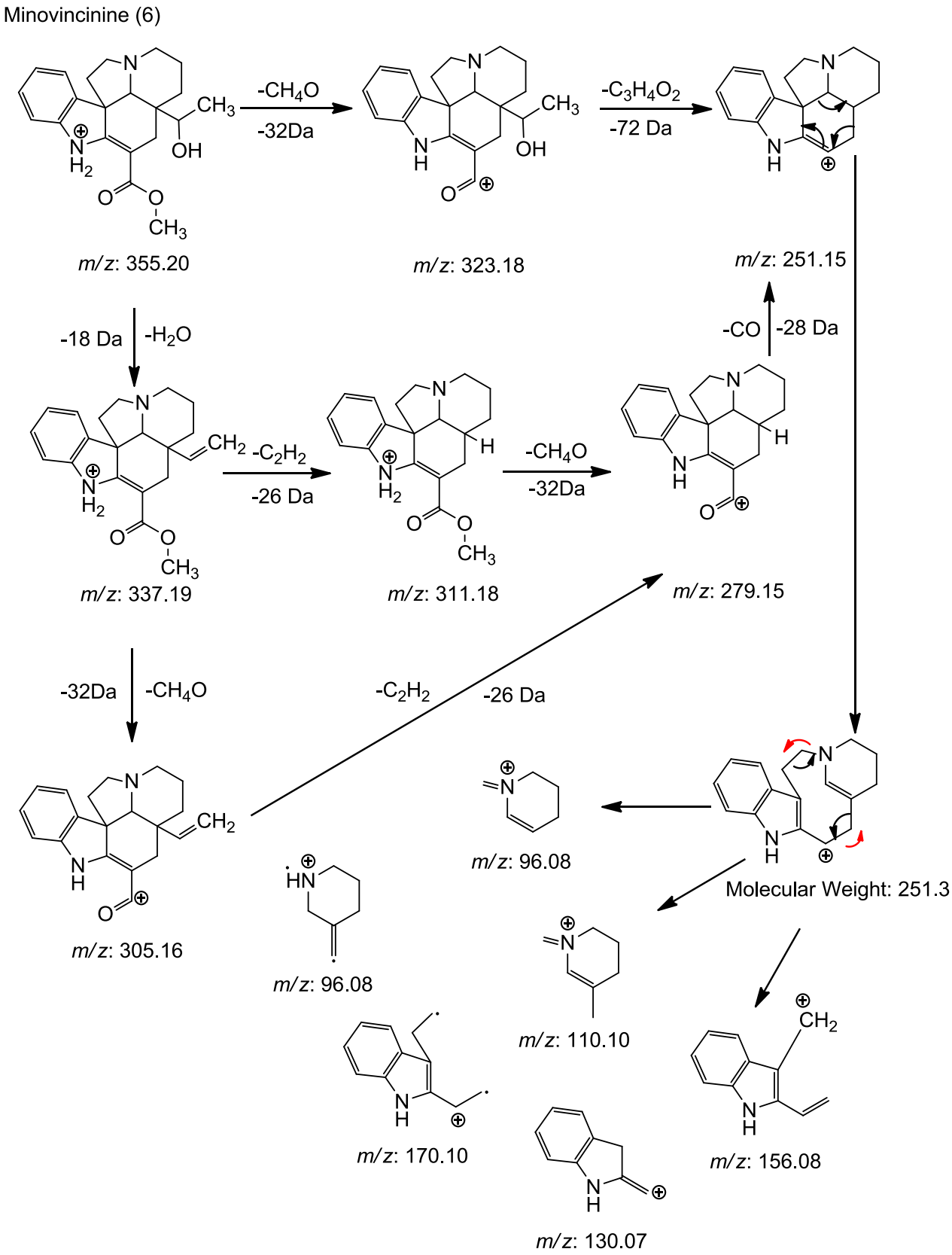


Figure 4- 16: Suggested scheme for fragmentation pattern of minovincinine (6).

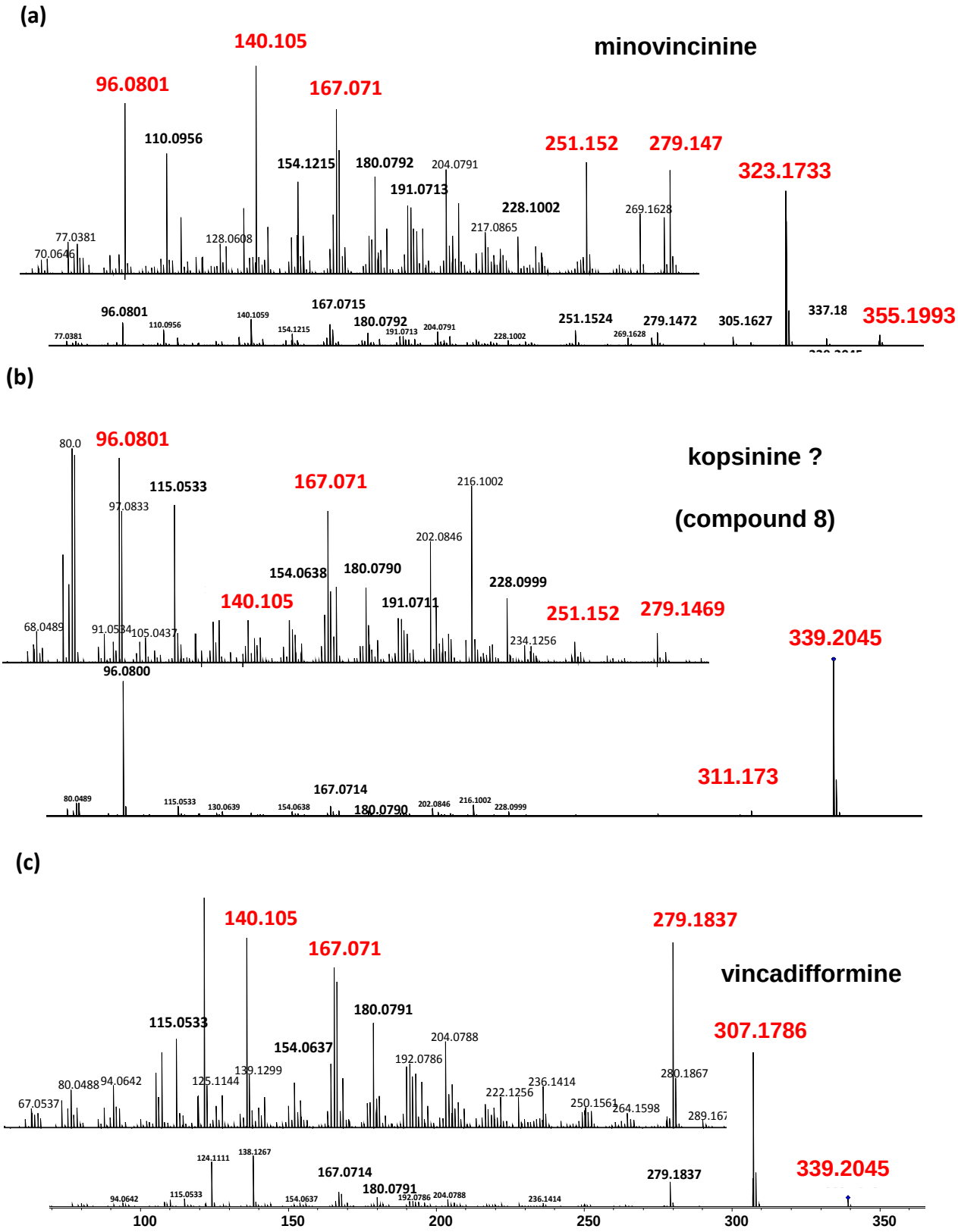


Figure 4- 17: MS-MS spectra for; (a) minovincinine; (b) compound (8); (c) vincadifformine (zoomed spectra).

4.3.3 Quantitative differences in the MIA contents of control and stressed *V. minor* leaves

To comprehensively describe the MeJA effect, a sound determination of the corresponding changes of the indole alkaloids concentrations are required. For this, a solid quantification of the MIAs presents in MeJA treated plants in comparison to the control plants is required. Unfortunately, due to the putative differences in UV absorbance, the peak area from the PDA detection cannot be used a reliable basis for any quantification. In contrast, the response intensities in the Total Ion Chromatogram (TIC) of the LCMS runs could be used for quantification, assuming that the analyzed substances have a similar ionization behavior. For the calculation of the peak areas, the Find-Molecular-Feature function of the Data Analysis Program (Bruker Daltonics) was used. Accordingly, further analyses of the MeJA treated as well as of the control plants were conducted by using LC-MS analyses. For this, the old leaves were extracted as mention in the Material and Method section. Strychnine was added as an internal standard to the solvent used in the extraction. After analysis of the samples by LC-MS, the peak areas are calculated. Subsequently, peak areas are normalized to the internal standard strychnine (Table 4-11).

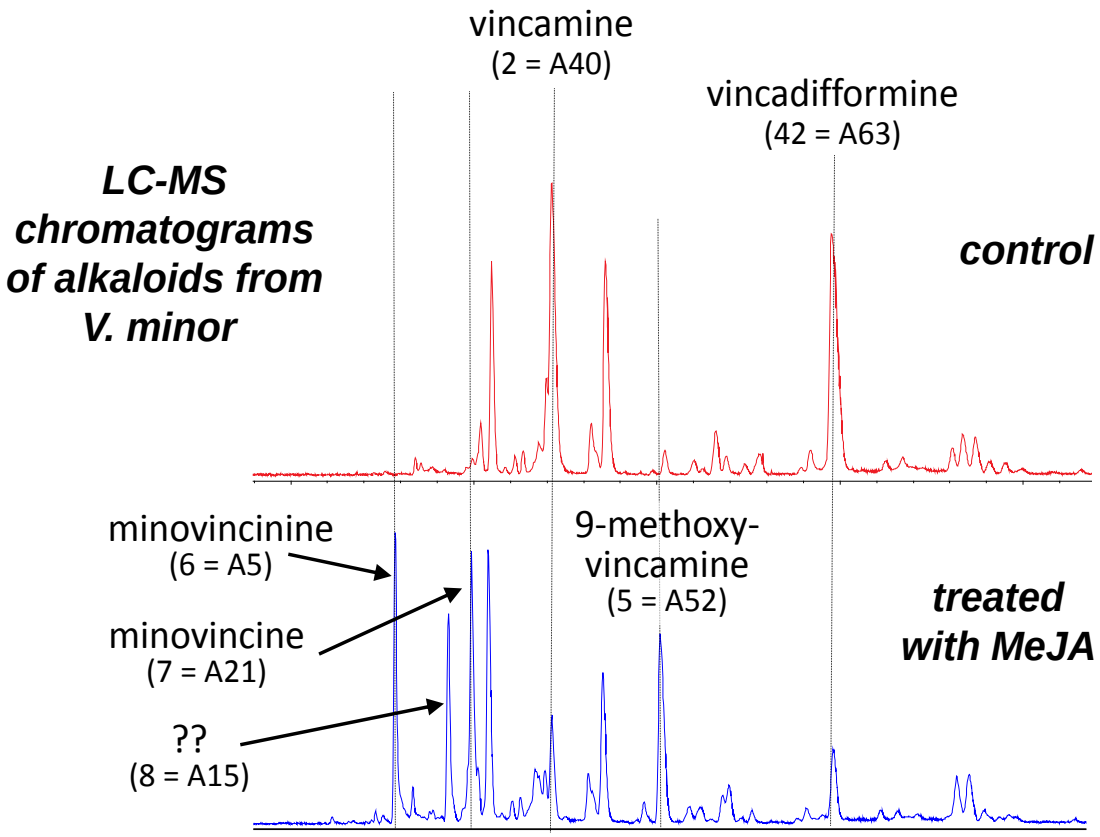
Based on the comparison of the peak areas in both samples when normalized to the internal standard, the relative changes (fold change) could be calculated. For this, the differences in the areas of a certain MIA between the MeJA treated and the control plants were divided by that of the control. Consequently, the relative change for the strychnine standard is zero. A value less than zero means that the concentration of this compound is lower in the treated plants than that in the controls; meaning that it decreased in response to the MeJA treatment. Values higher than zero state that the amount of this certain compound is higher in the treated sample than in the control, signifying that the concentration of the compounds increased in response to the MeJA treatment. In all cases, in which no peak area couldn't be calculated (i.e., due to the absence of any peak or because its value was below the detection limit), no value was calculated. The corresponding data are compiled in Table 4-11. In order to prevent a mixing of the indication with the numbering of the nine compounds mentioned above, the compounds were termed A1 to A68.

It clearly could be recognized that the concentrations of most of the compounds are changed in response to the MeJA treatment. Surprisingly, many substances show the same fold change value. We can conclude that these substances are converted in a similar manner, mentioning that the mode of conversion either is the same or is related to the same pathway.

Table 4- 11: Changes in the alkaloid composition of old leaves from MeJA treated *V. minor* plants in comparison to the control.

Compound	RT [min]	Mass *m/z* $[M+H]^+$	Area		Fold change
			Control	MeJA	
A-1- ercinamine	6.12	369.1814	290213	713002	1.4568
A-2	6.37	399.1899		11757	
A-3	7.05	339.1702	25261		
A-4 - strychnine - internal standard	7.55	335.1754	2265468	2265468	0.0000
A-5- minovincinine	7.78	355.2017	690832	3967222	4.7427
A-6	7.87	387.1915	17734		
A-7	7.98	351.1705	237845	159802	-0.3281
A-8	7.95	353.186	135482		
A-9	8.2	369.1809	111127	282113	1.5387
A-10	8.21	371.1597	9146		
A-11	8.27	461.2272	8679		
A-12	8.43	351.1706		66024	
A-13	8.68	371.1963		28844	
A-14	8.73	367.1648		29523	
A-15 kopsinine? (8)	9.11	339.2072	269042	1596549	4.9342
A-16	9.15	369.1808		124189	
A-17	9.21	341.1864	94954		
A-18	9.5	383.1971	361767	747430	1.0661
A-19	9.57	355.2012	12328		
A-20	9.61	369.181	90157	634032	6.0326
A-21- minovincine	9.73	353.1865	746834	3840636	4.1426
A-22	9.65	321.1601		45373	
A-23	9.82	197.1172	30277	34279	0.1322
A-24	9.82	413.2071	18002	41205	1.2889
A-25	9.79	321.1601		171255	
A-26	9.86	355.2017	93520		
A-27	10.06	299.2115		31033	
A-28	10.2	355.2017	3264562	4931241	0.5105
A-29	10.18	353.1856	60543		
A-30	10.26	399.1916	104693		
A-31	10.43	383.1964		101943	
A-32	10.67	429.2025	98505		
A-33	10.76	371.1969	99686	163620	0.6414
A-34	10.96	353.1858	55324	85790	0.5507

Compound	RT [min]	Mass *m/z* $[M+H]^+$	Area		Fold change
			Control	MeJA	
A-35	11.24	369.1797		34402	
A-36	11.32	281.2019	1263299	994446	-0.2128
A-37	11.32	299.2124	221377	161857	-0.2689
A-38	11.29	351.1696		119096	
A-39	11.4	383.1969	148865	436987	1.9355
A-40- vincamine	11.66	355.202	11889948	2166515	-0.8178
A-41	11.66	337.1911	125178		
A-42	11.66	745.3726	45245		
A-43	11.66	747.3713	10454		
A-44	11.75	399.191		302515	
A-45	11.99	369.2172		98857	
A-46	12.64	385.2129	614505		
A-47	12.7	351.1705	56374	185933	2.2982
A-48	12.93	339.1702	222699	138744	-0.3770
A-49 - strictamine	13.14	323.1758	2158369	1943188	-0.0997
A-50 - methoxyvincamine	14.07	385.2123	97681	769062	6.8732
A-51	14.51	341.1862	447273	393610	-0.1200
A-52 - 9-methoxyvincamine	14.58	385.2125	1065412	6041931	4.6710
A-53	14.51	805.3912		5897	
A-54	15.32	323.1754	269266	200044	-0.2571
A-55	15.77	401.2071	155801		
A-56	15.86	369.1813	747136		
A-57	16.16	323.1762	776500	850393	0.0952
A-58	16.29	338.1811		338742	
A-59	16.93	323.1755		120218	
A-60	16.97	397.1762	238746		
A-61	17.65	339.2072	168451		
A-62	18.3	355.2022	319555		
A-63 - vincadifformine	19.1	339.2074	1687203	793954	-0.5294
A-64	20.41	354.1944		249623	
A-65	22.38	369.2168	304875	208057	-0.3176
A-66	22.43	353.2222	959790	1266222	0.3193
A-67	22.92	355.2375	260067	265583	0.0212
A-68	23.43	369.2166	550914	205161	-0.6276



The numbering of alkaloids mentioned in Table 4-6 is added to the chromatograms presented in as Figure 4-9.

Unfortunately, for most of the observed changes – until now – no prediction on the exact molecular structure and the biosynthetic pathway involved could be made. However, based on the structural similarities of vincamine and 9-methoxyvincamine and their inverse changes in concentrations (which already had been presented in Figure 4-9), it is postulated that vincamine serves a precursor for 9-methoxyvincamine (Figure 4-18). In the same manner, it could be deduced that vincadifformine is converted first to minovincinine and subsequently to minovincine (Figure 4-18). Because of the similarities to other modifications of secondary metabolites (Rodriguez *et al.*, 2003; Giddings *et al.*, 2011), it is likely that the corresponding reactions are catalyzed by cytochrome P450 enzymes. An unequivocal confirmation for the occurrence of such conversions should be provided by classical pulse-chase experiments using ^{13}C or ^{14}C labeled alkaloids, which could be generated by feeding the corresponding labeled precursors (tryptophan) to *V. minor* plants before MeJA treatment.

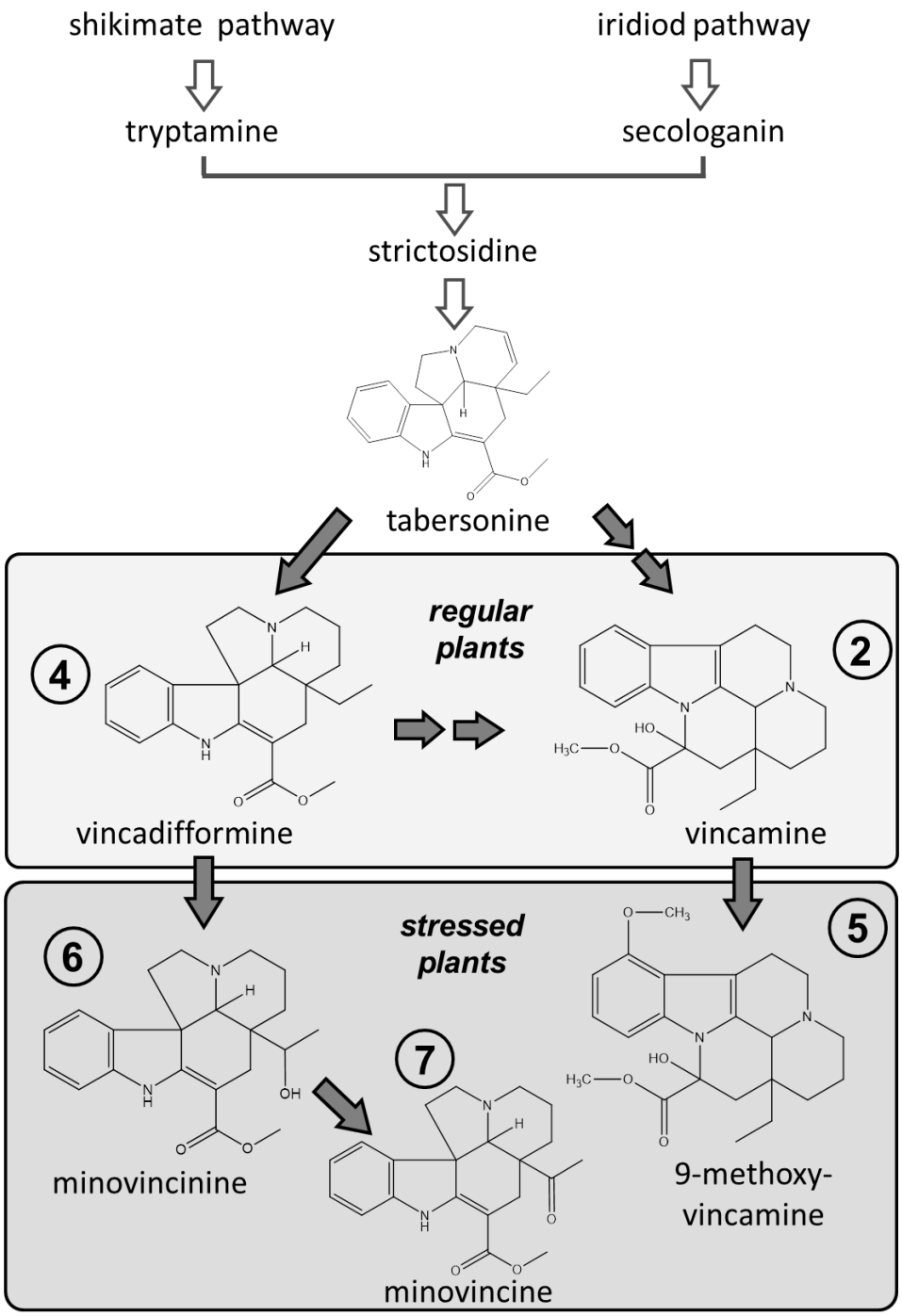


Figure 4- 18: Postulated conversion of indole alkaloids in *V. minor* in response to MeJA treatment.

Summing-up – 4.3 The effect of MeJA on the pattern of indole alkaloids of V. minor

MeJA treatment causes a significant shift in the alkaloid pattern. Based on the reverse changes, it is deduced that vincadifformine is transformed first to minovincinine and finally to minovincine. In the same manner vincamine is converted to 9-methoxyvincamine (Figure 4-18). The identification of the enzymes involved in the conversion steps, appears to be an attractive topic for further studies.

The discovery of 9-methoxyvincamine as a novel natural product further outlines that even well-known, extensively studied plants such as *V. minor*, might be an auspicious source for the generation of novel phytochemical drugs, when grown under defined stress conditions.

4.4 Application of different growth regulators on *V. minor*

The various indole alkaloids present in *V. minor* extracts reveal quite different biological activeties (Khanavi *et al.*, 2010; Hasa *et al.*, 2013). Accordingly, the alkaloid patterns and concentrations determine the related biological potency, and thus, also the corresponding pharmacological effectivity. As outlined above, the treatment with MeJA strongly alters the alkaloid composition in *V. minor*. In order to elucidate the extend of these changes and also to investigate whether other growth regulators (e.g., salicylic acid, hydrogen peroxide) have similar effects, further experimental approaches have been conducted. Apart from growth regulators, also resveratrol was applied to the *V. minor* plants. This stilbene is known to inhibit oxidative enzymes (Fan and Mattheis, 2001; Kutil *et al.*, 2014) and thus, might interfere with the indole alkaloid biosynthesis, too. These studies – in combination with the data revealed from the experiments, in which MeJA was applied – should indicate, if and to which extent the alkaloid pattern of *V. minor* could be modulated by exogenous factors.

In the course of these experiments, the growth regulators had been applied twice; the second application was performed two days after the first treatment. Resveratrol was applied four times via the irrigation water. Leaves – separated to young and old ones – had been collected four times after the treatment (for details see material and method section). In order to estimate putative losses during the entire extraction and quantification process, strychnine was added as an internal standard to the solvent used in the first extraction step. In the same manner, the authentic alkaloid solutions (vincamine and vincadifformine) used for quantification were prepared using the identical solvent containing the internal standard. Accordingly, the ratio of peak areas between the analyte and internal standard could be used to quantify the

concentrations of these alkaloids in the various samples. However, a major problem with respect to the quantification of the other alkaloids (which were not available as standard substances) corresponds to the fact that these alkaloids may reveal quite different absorbance spectra. For instance, vincamine displays a higher absorbance at 280 than vincadifformine, whereas the latter one reveals a far higher absorbance at 330 nm than vincamine. Consequently, it is quite problematic (and nearly impossible) to soundly calculate the concentration of a certain alkaloid, whose absorbance is not known. Indeed, according to the literature, minovincine and minovincine possess quite similar UV spectra as vincadifformine; consequently, they could be quantified based on the response factor of vincadifformine. In the same manner, epivincamine and methoxyvincamine - due to their similar absorbance properties as vincamine - could be quantified based on the vincamine calibration curve. For all other alkaloids, putative differences in their concentration could only be determined as relative values.

4.4.1 Effect of growth regulators on growth and development of *V. minor*

The application of growth regulators and resveratrol, respectively, did not change the performance of the *V. minor* plants (Figure 4-19).

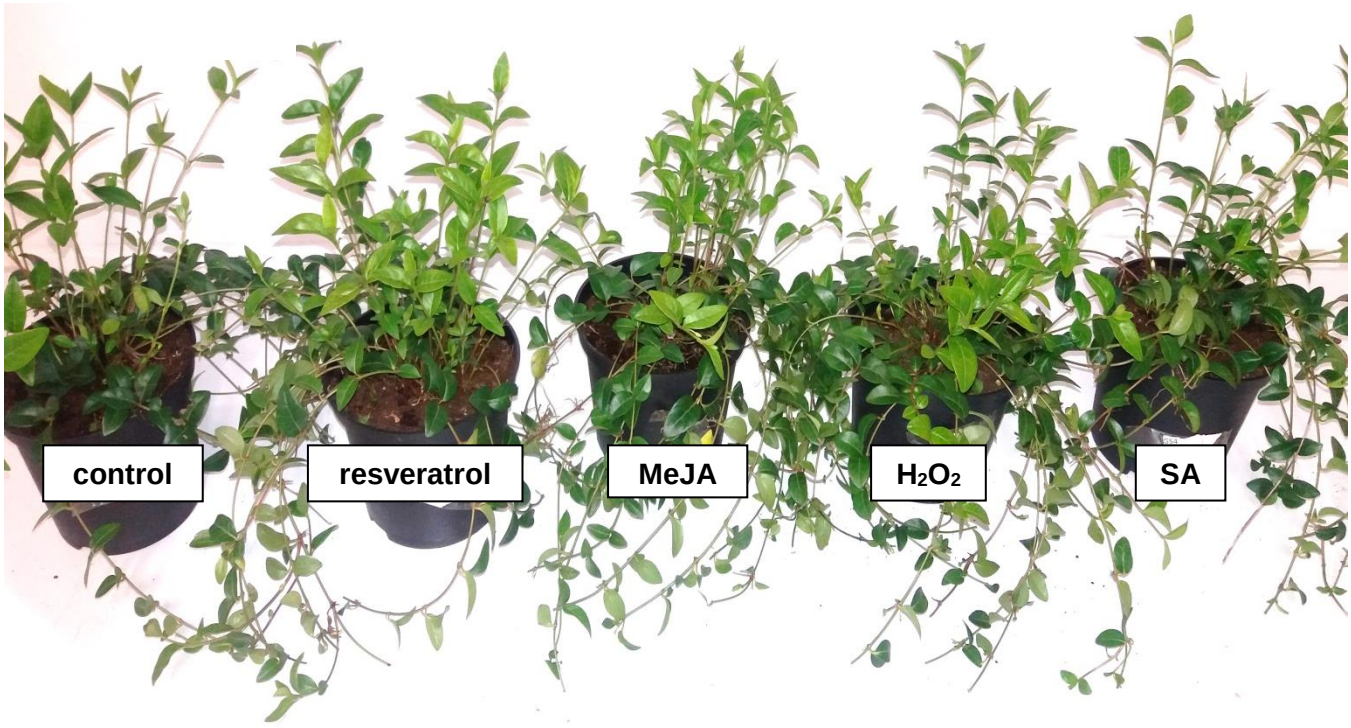


Figure 4- 19: *V. minor* plants treated with different growth regulator: Abbreviations: MeJA (methyl jasmonate); H_2O_2 (hydrogen peroxide); SA (salicylic acid).

In contrast to the habitus, the appearance of individual leaves of treated and control plants differs significantly. Whereas the leaves of control plants show the typical healthy and vital appearance, those of the treated plants reveal typical stress symptoms (Figure 4-20).

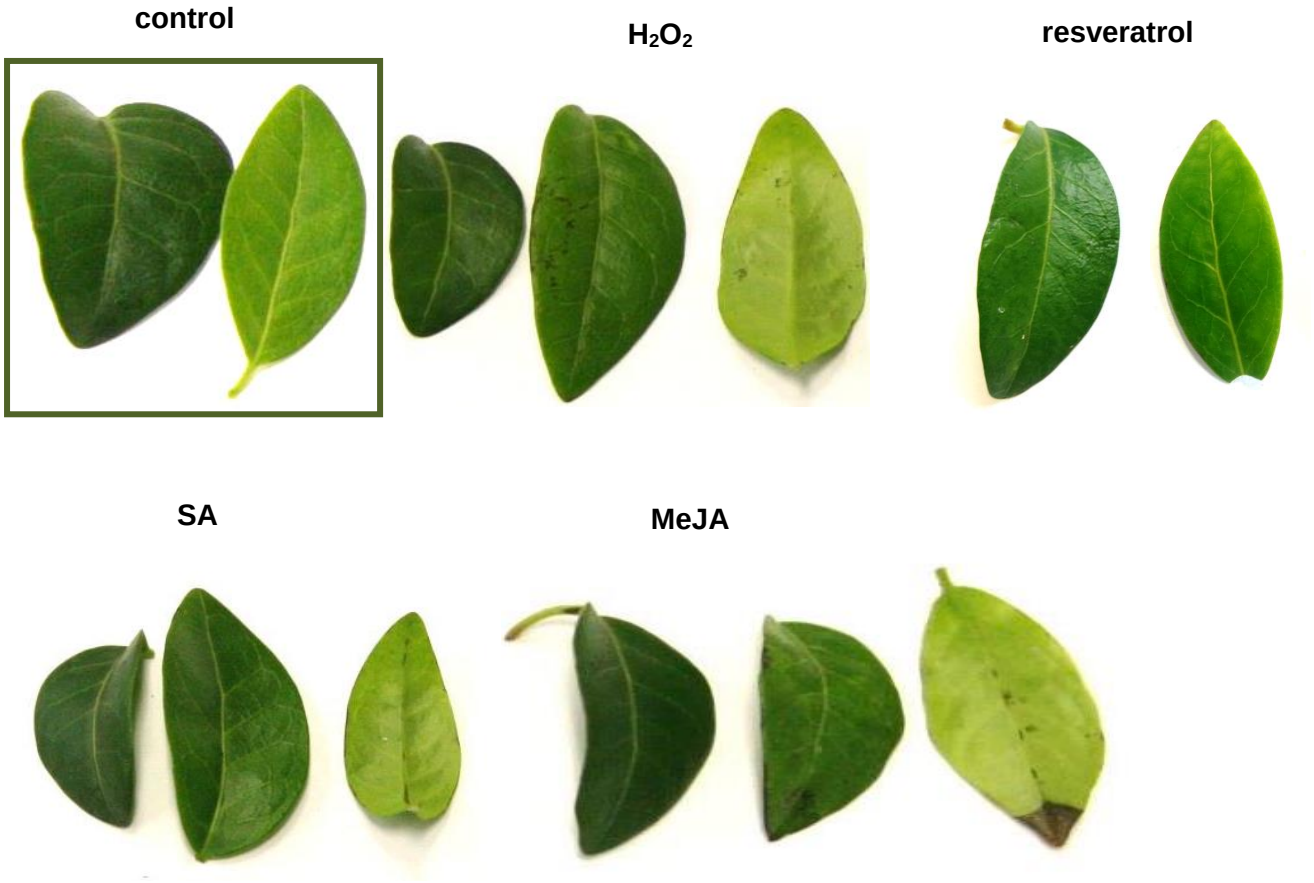


Figure 4- 20: Leaves of *V. minor* plants treated with a different growth regulator

The most significant difference of the treated plants in comparison to the control concerns the young leaves: independently to the compound applied, many young leaves exhibit several necrosis. On the contrary, in old leaves, these stress symptoms are much rare and their extent is quite smaller. In Figure 4-21 these typical differences are displayed exemplarily.

Figure 4- 21: Characteristic differences of leaves from control and stressed plants.

4.4.2 Effect of growth and development on the content and composition of indole alkaloids in *V. minor*

As already mentioned, in old leaves of *V. minor*, vincamine represents the major alkaloid. Yet, in young leaves, vincadifformine turned out to represent the alkaloid exhibiting the highest concentration (Figure 4-1, page 44). Consequently, before the effect of the various growth regulators were analysed in detail, the influence of leaf age on the alkaloid content was quantified.

In the controls, the concentration of vincadifformine is markedly lower in old leaves than in young ones (Figure 4-22), putatively due to the massive gain of biomass per leaves[3]. From this, it could be argued that no further biosynthesis of vincadifformine occurs in old leaves. However, due to the dynamic equilibrium of biosynthesis, degradation, and conversion of the indole alkaloids, the situation is much more complex and will be stressed in detail in the next chapter (discussion section: 5.1). In contrast, the concentration of vincamine is markedly higher in old leaves (Figure 4-22). Accordingly, the total content of vincamine per leaf increases in the course of leaf maturation, suggesting that this alkaloid is synthesized also in old leaves.

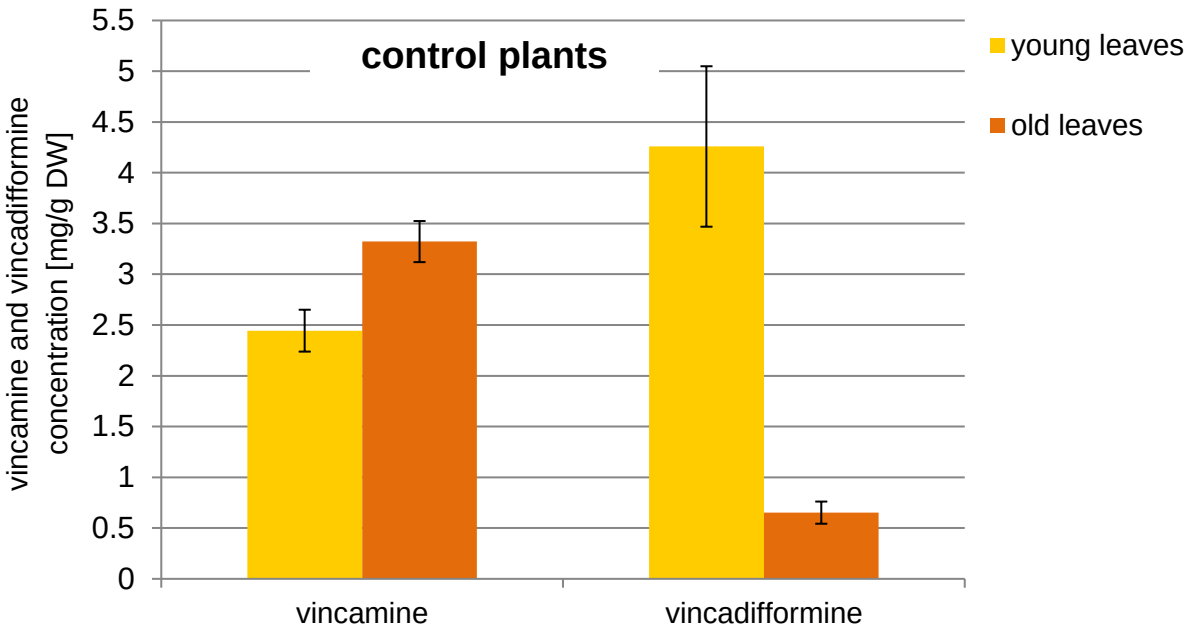


Figure 4- 22: Concentration of vincamine and vincadifformine of young and old leaves. Values correspond to the average of all control plants analysed in this part of the investigation.

[3] When the content of a certain alkaloid is identical in young and in old leaves, - due to the massive gain the leaf weight (fourfold) - the alkaloid concentration in old leaves will be markedly lower (about one forth).

These data clearly show that a sound assignment of the developmental stage of the leaves used for the scheduled experiments is required. Accordingly, for all approaches quantifying the alkaloid concentration in differentially treated plants, always a differentiation between old and young leaves had been made.

The various growth regulators had been applied two times to *V. minor* plants during the blooming phases. The leaves were collected at four different time intervals (1, 4, 8, and 14 days after the treatment). As already mentioned for MeJA, also the application of salicylic acid and H_2O_2 impact the pattern and the concentration of the alkaloids.

4.4.3 Effect of growth regulators on the composition of indole alkaloids in *V. minor*

Qualitative changes in response to salicylic acid

The application of salicylic acid (SA) induced only minor changes in the concentration of MIAs[4] (see below 4.4.4) but did not cause any qualitative changes, i.e., SA did not elicit the generation of new alkaloids.

Qualitative changes in response to H_2O_2

In contrast to the treatment with SA, which indeed altered the only concentration of alkaloids, but did not induce major changes in their composition of MIAs the treatment with H_2O_2 significantly impacts on both, alkaloids concentration and their spectrum. As outlined in the scientific background (section 2.3.4.1) H_2O_2 is involved in the induction of phytoalexin biosynthesis in the course of host-pathogen interactions, frequently denoted as oxidative burst (Wojtaszek, 1997), and in numerous signalling events by interacting with other signal molecules, such as JA, SA, ethylene, Ca^{++}, NO etc. (for review see (Saxena *et al.*, 2016). Previous studies showed that exogenous application of H_2O_2 induced indole alkaloid biosynthesis in *C. roseus* (Tang *et al.*, 2009). In addition, in hairy roots cultures of *V. minor*, H_2O_2 increased the total alkaloid content (Verma *et al.*, 2014b). However, the authors did not analyze the entire alkaloid pattern. Accordingly, the application of H_2O_2 to *V. minor* plants seemed to be very promising and it was integrated into this study, too.

[4] In case of SA no significant change in the spectrum of the alkaloids was observed; except for single plant after 14 days, which showed a drastic decrease in 9-methoxyvincamine, whereas minovincinine and minovincine were present in high concentration. Since the reasons for this particular and unique finding are unknown, it remained unconsidered in the related deductions (Appendix: Figure A-66).

In addition to the changes mentioned to be induced by a MeJA treatment, further modifications of the alkaloid composition were detected when H_2O_2 was applied. These differences mainly concern 9-methoxyvincamine and a so far, unknown alkaloid, denoted **10** in Figure 4-23.

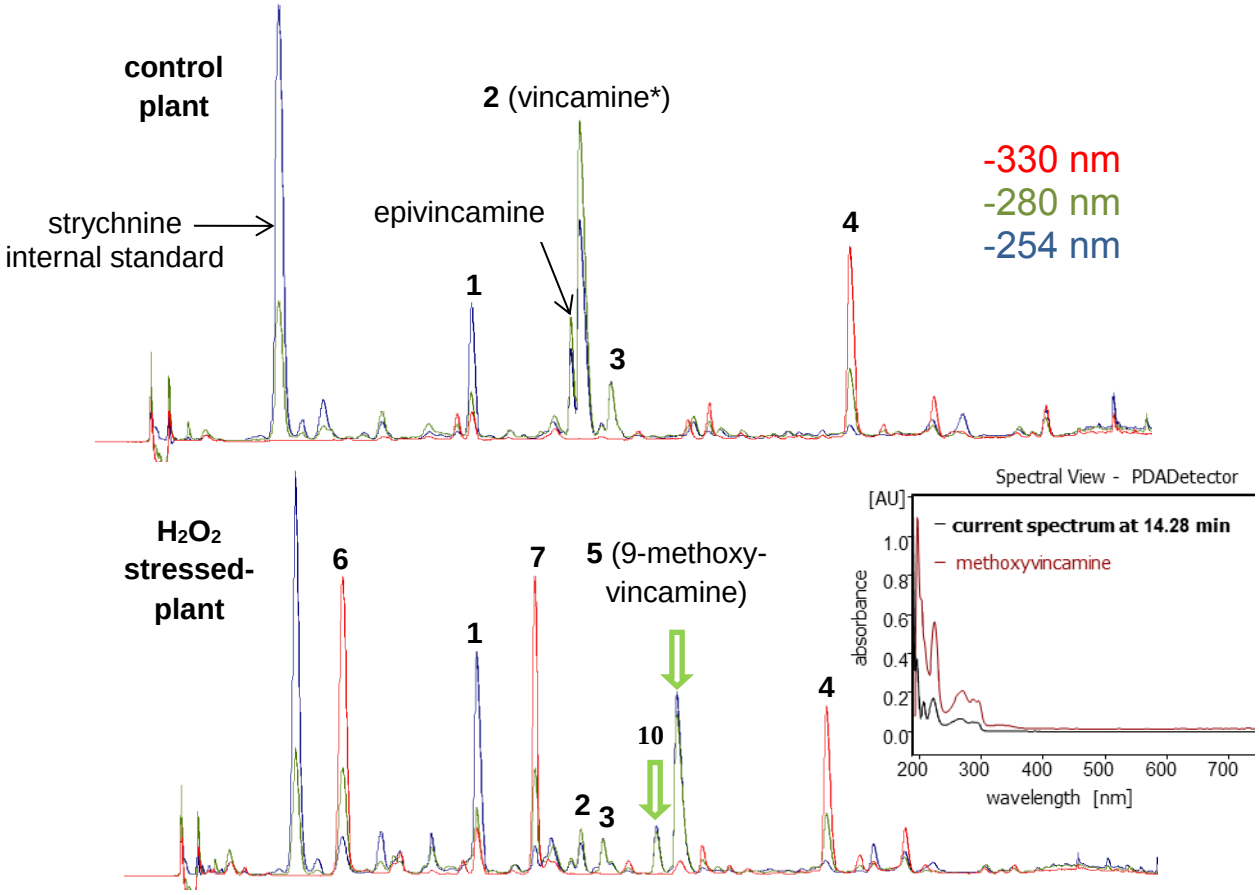


Figure 4- 23: HPLC chromatogram of the old leaves treated with H_2O_2. The stressed plant contains two peaks (green arrows) have the same UV spectrum of methoxyvincamine with a comparison to control. Compounds are represented by numbers: 10-hydroxycathafoline (1); strictamine (3); vincadifformine (4); minovincinine (6). minovincine (7). *As mentioned above, due to the problems in separating vincamine and epivincamine, always the sum of these both alkaloids are denoted as vincamine.

Compound 10:

In the same manner, as a massive increase of the 9-methoxyvincamine concentration could be detected in MeJA treated plants, the concentration of the unknown substance was considerably enhanced in H_2O_2 treated plants. Astonishingly, the UV-spectrum of this unknown substance was very similar to that of 9-methoxyvincamine. In order to get further information on this compound, the samples were re-analyzed using LC-MS. It turned out that the substance responsible for the unknown peak reveals a molecular weight of 384, which is identical to that of 9-methoxyvincamine. In order to find out, if there might be a metabolic relation between this unknown substance and 9-methoxyvincamine, a further thorough analysis of the chromatogram of the control plants was performed.

When checking the extracted ion chromatogram (EIC) of all peaks revealing a mass identical to methoxyvincamine ($M+H^+$ = 385 *m/z*), it turned out that several compounds are present in control as well as in the stressed plants that reveal this molecular mass (Figure 4-24).

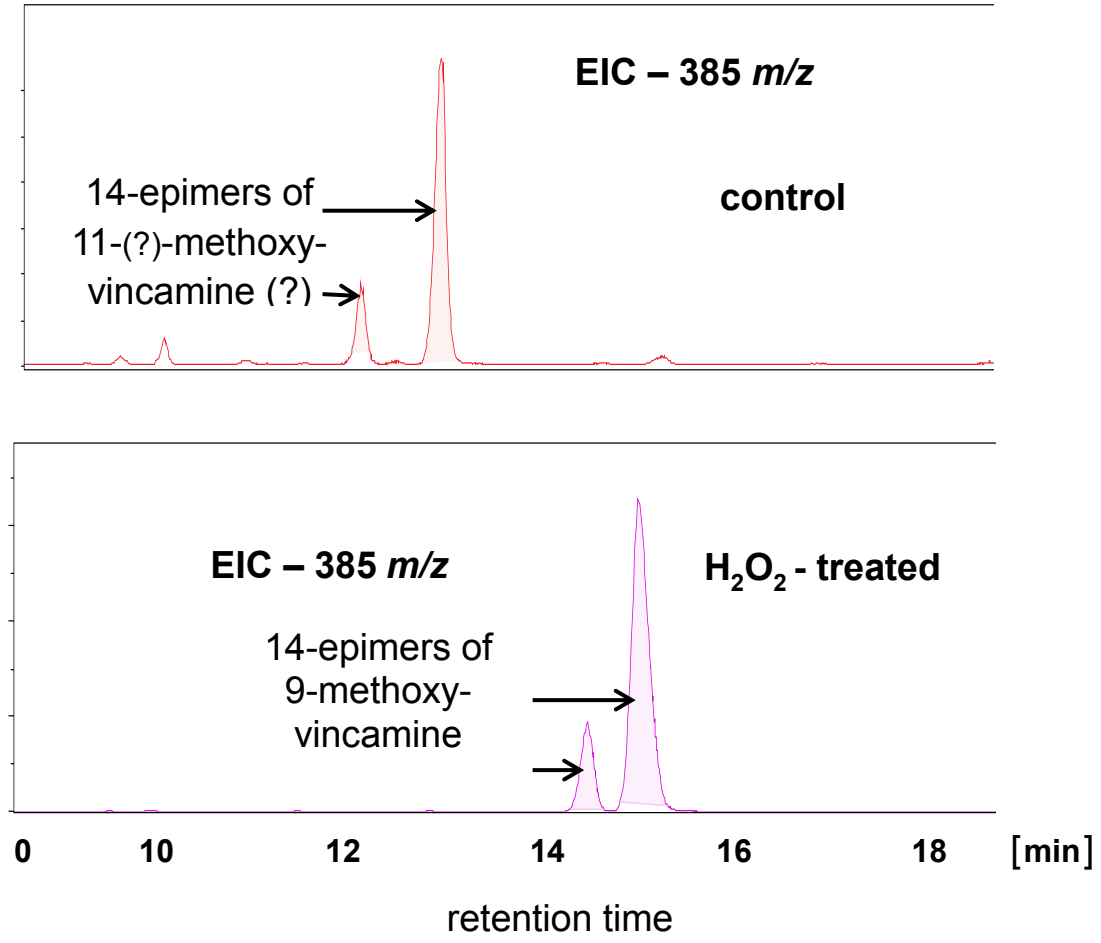


Figure 4- 24: The extracted ion chromatograms (EIC of 385 *m/z*). In both samples, control leaves and H_2O_2 treated leaves, the mass of 385.21 (corresponding to methoxyvincamine) is present as double peaks. Has to be mentioned that the expansion of both samples strongly differs.

Interestingly, two main double peaks could be detected in the control as well as in the stressed leaves; but their retention time differs by about 3 min (Figure 4-24). Nonetheless, the MS-MS fragmentation patterns of these four compounds are very similar, except the intensity of the fragment (367 *m/ z*, corresponding to $[M -18+H]^+$; Figure 4-25, Table 15).

As 11-methoxyvincamine was already reported to occur in *V. minor* (Štrouf, Trojánek 1964; Szabó *et al.*, 1996), it can be concluded that the compounds eluting between 12 and 13 min correspond to this compound, whereas the substances eluting between 14 and 15 is 9-methoxyvincamine. Like it was reported for stereoisomers having an eburnane basic skeleton (e.g. vincamine, eburnamine), the observed double peaks for 11- and 9-methoxyvincamine, respectively, might be due to epimers at C-14 (Kováčik and Kompiš, 1969; Czira *et al.*, 1984), i.e., in methoxyvincamine and methoxy-14-epivincamine.

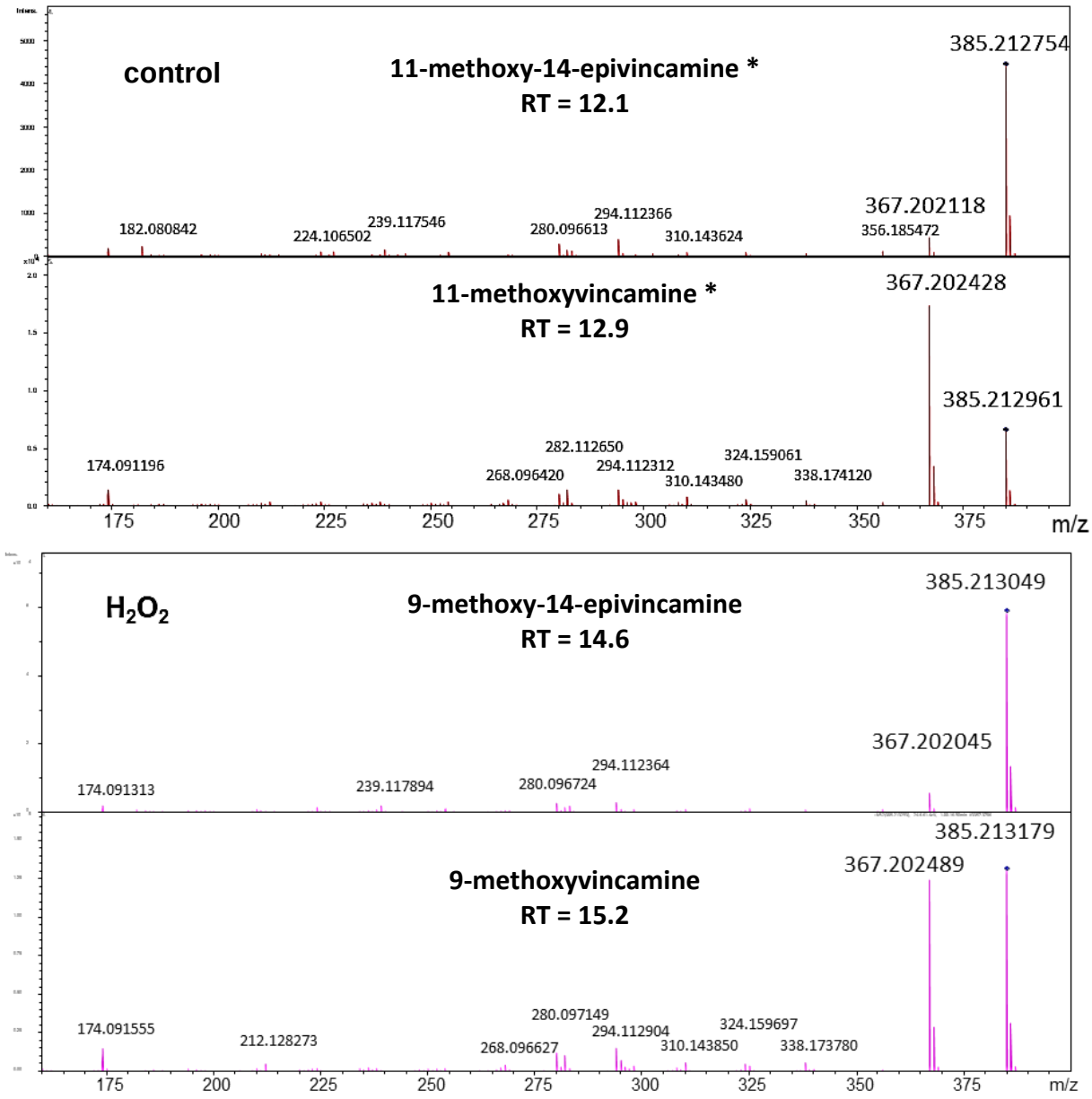


Figure 4- 25: MS-MS spectra of the double peaks in control and H_2O_2 treated plants. The fragmentation pattern of the two isomers is different, especially in the intensity of 367m/z; yet the abundance of other fragments is nearly the same. * The methoxy group at 11-position in the indole ring was reported by Štrouf and Trojánek, (1964) and Szabó et al. (1996).

As outlined above, in stressed plants, methoxyvincamine is derived from the vincamine (Figure 4-18). In this manner, the epimers of both methoxyvincamine isomers mentioned above should be derived from corresponding 14-epimers of vincamine; i.e., vincamine and 14-epivincamine. Accordingly, an EICs for the mass of vincamine ($M+H^+$ = *m*/*z* 355) was generated for control *V. minor* extracts (Figure 4-26). The extracted ion channel (*m*/*z* 355) showed three major peaks. Yet only two revealed the same fragmentation pattern of vincamine, representing 14-epivincamine and vincamine (Figure 4-27); the other peak eluting at about 10 min, about 10 min, corresponding to 10-hydroxycathofoline.

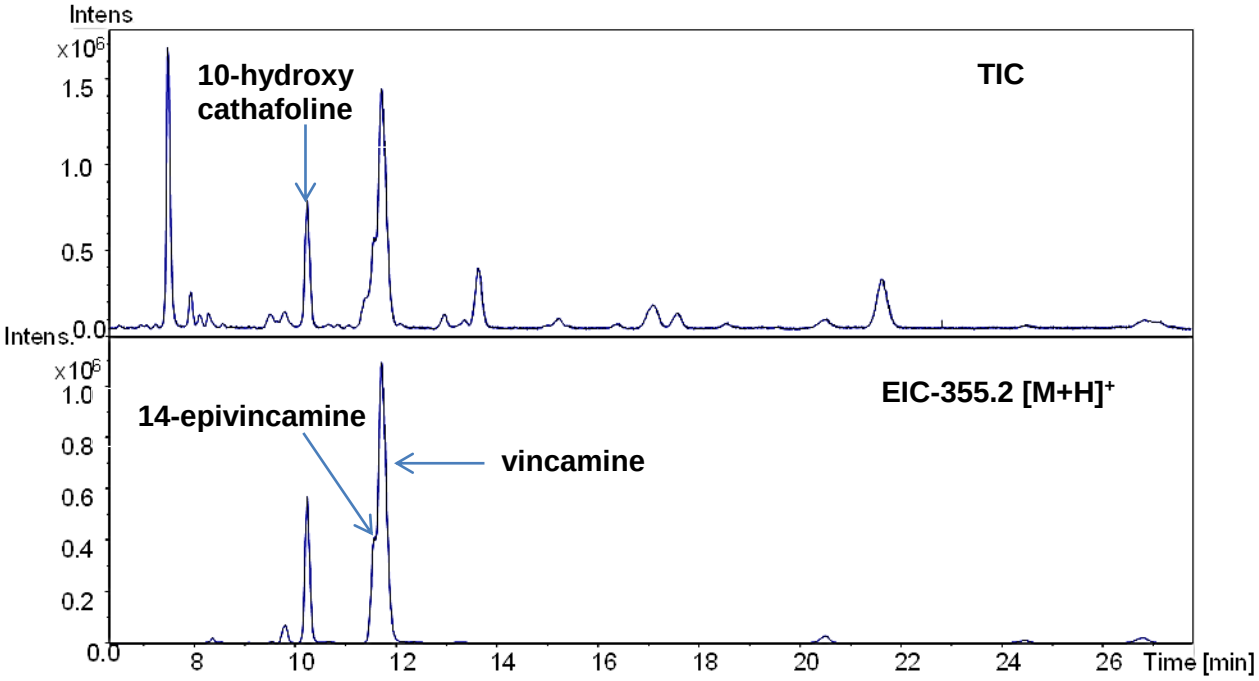


Figure 4- 26: LC-MS chromatograms – TIC and EIC 355.2 [M+H]⁺. The total ion chromatogram (TIC) outlines all alkaloids present in the control sample, whereas the extracted ion chromatogram (EIC) only displays those revealing a mass of 355.2 ($M+H^+$). As 14-epivincamine cannot be fully separated from vincamine, it is detectable just as a shoulder of the vincamine peak.

With respect to the fragmentation pattern of both vincamine related peaks, we have to consider that in the MS-MS-spectrum – apart from the $[M+H]^+$-fragment (=355 *m*/z) - the most abundant mass corresponds to the dehydrated molecule. It is noteworthy to mention that the different stereochemistry at C-14 influences this loss of water, resulting in different intensities of the $[M-18+H]^+$-fragment (=337 *m*/z; Figure 4-27; (Czira *et al.*, 1984; Kováčik and Kompiš, 1969)). Due to the higher stability of the equatorial hydroxyl group, the abundance of the 337 *m*/z-fragment is quite lower in 14-epivincamine than in vincamine, which exhibits an axial (more unstable) hydroxyl group ((Czira *et al.*, 1984; Saxton, 1983b); Figure 4-27, Table 4-12).

Table 4- 12: Relative abundance (%) of the main fragments of vincamine, methoxyvincamine, and their 14-epimers.

RT min	Compound	$[M+H]^+$	%Relative abundances			
			$[M+1]^+$	[M-18+1]	*m/z* 174	*m/z* 144
11.4	14-epivincamine	355.2025	100	16.1	0	5.3
11.7	vincamine (2)	355.2027	100	76.6	0	9.9
12.1	11-methoxy-14-epivincamine?	385.2128	100	10.4	4.8	0
12.9	11-methoxyvincamine?	385.2129	37	100	7.9	0.2
14.6	9-methoxy-14-epivincamine (10)	385.21304	100	9.7	3.4	1
15.2	9-methoxyvincamine (5)	385.2132	100	95.2	11.2	2.8

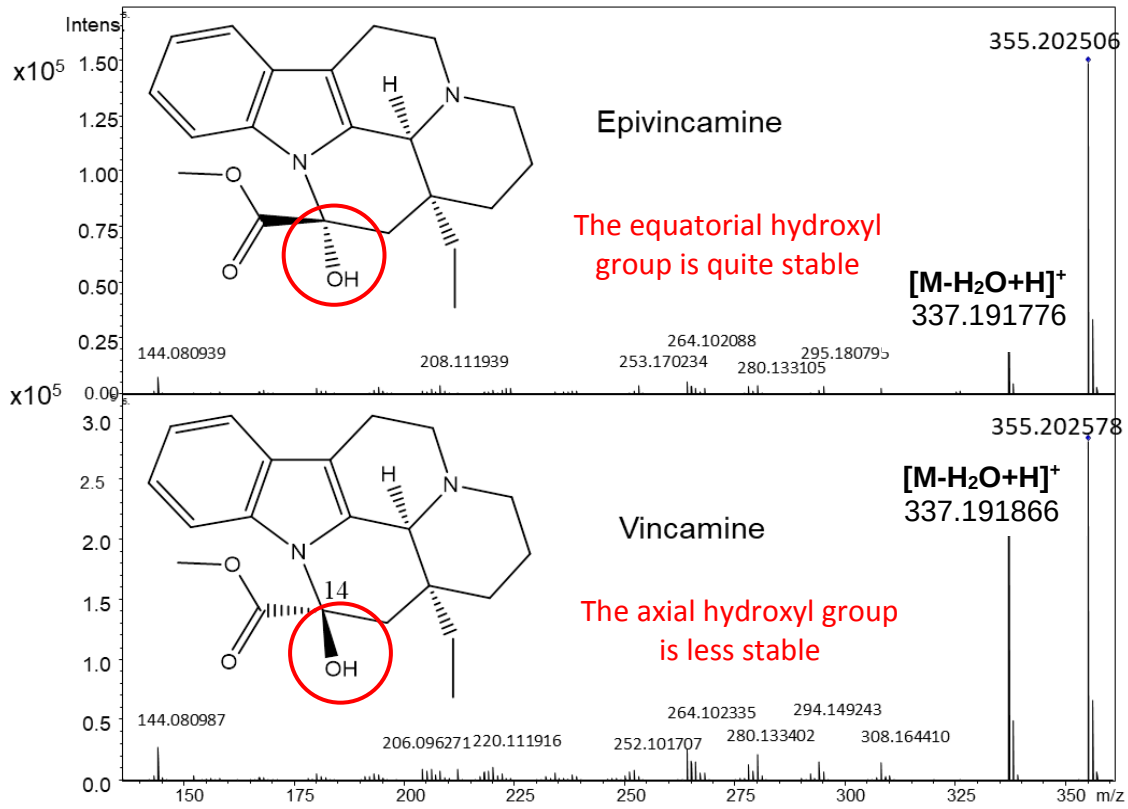
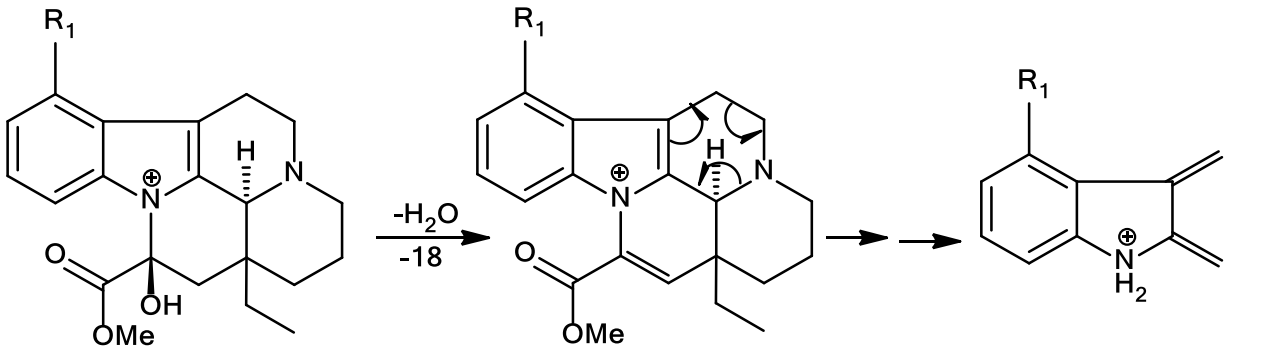


Figure 4- 27: MS-MS spectra of the two peaks with RT 12 min in control sample. The fragmentation patterns are very similar, except the difference in the intensity of the first fragment (337 m/z) (like in case of the methoxyvincamine).

In the same manner, it can be explained that the methoxy-14-epivincamines are more stable with respect to a dehydration than the methoxyvincamine; a fact that properly explains the differences in the intensity of their fragmentation pattern (Figure 4-25). In both cases, the $[M-18+H]^+$-fragment (=367 *m*/z) has a higher abundance in the fragmentation pattern of the methoxyvincamines than in the case of the methoxy-14-epivincamines (Figure 4-28; Table 4-12).



2: (R_1=H) *m/z* 355
5: (R_1= OCH_3) *m/z* 385

2: (R_1=H) *m/z* 337
5: (R_1= OCH_3) *m/z* 367

2: (R_1=H) *m/z* 144
5: (R_1=OCH_3) *m/z* 174

Figure 4- 28: Main fragments of vincamine (2) and methoxyvincamine (5).

In conclusion, is can be deduced that in leaves of *V. minor*, methoxyvincamine is derived from vincamine, and methoxy-14-epivincamine from 14-epivincamine, respectively. However, we have to conclude that the position of methoxylation depends on the physiological state of the plant. Whereas 11-methoxyvincamine and its 14-epimer are present in the healthy and unstressed control plants, 9-methoxyvincamine and its 14-epimer are produced in stressed leaves.

Compound 11 and 12:

Up to now, the presence of a putative intermediate, 9-hydroxyvincamine could not be established; however, some evidence is already available. When checking the extracted ion chromatogram (EIC) of all peaks revealing a mass of hydroxyvincamine ($M+H^+$ = 371 *m/z*), it turned out that several compounds are present in control as well as in the stressed plants showed this molecular mass (Figure 4-29).

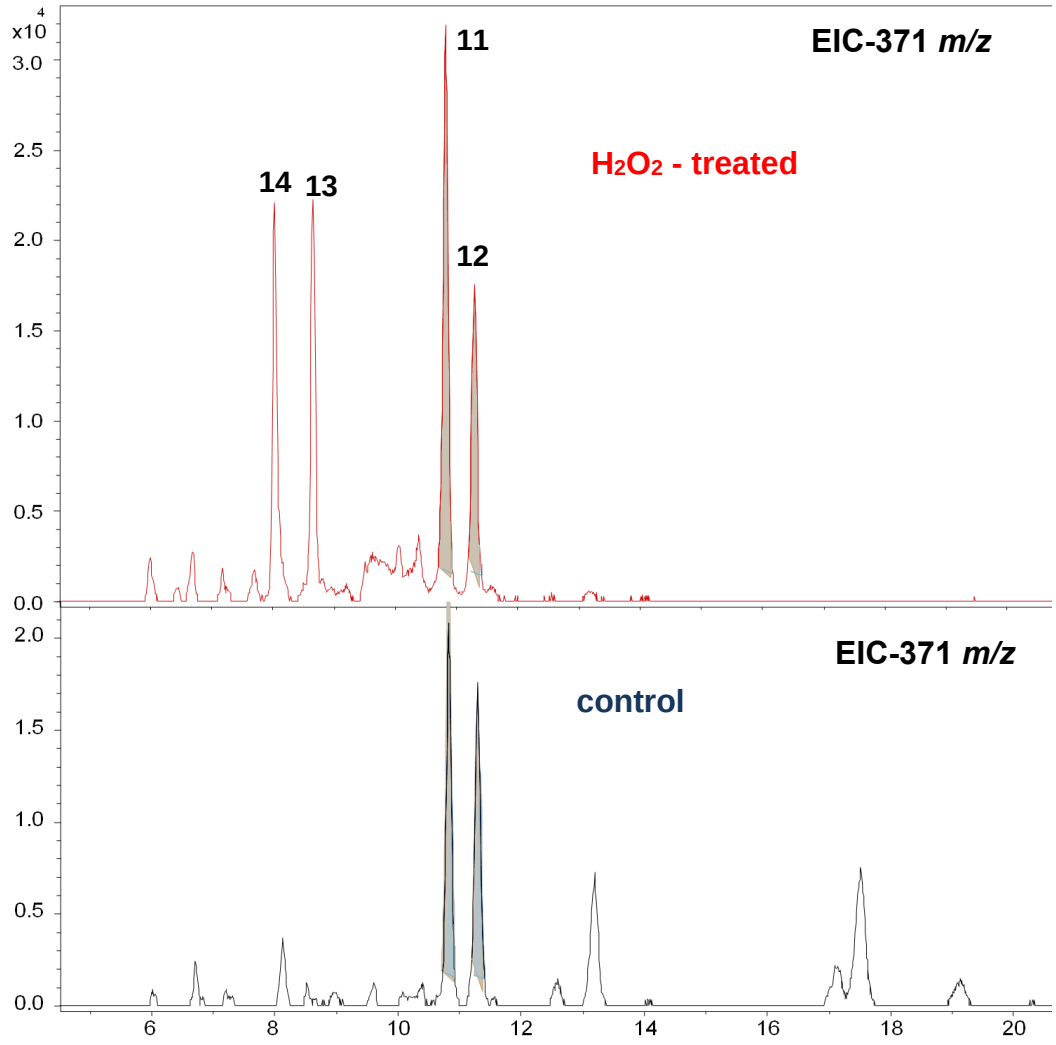


Figure 4- 29: The extracted ion chromatograms (EIC of 371 *m/z*) of old control and H_2O_2 treated leaves. The major unknown alkaloids are denoted 11, 12, 13 and 14.

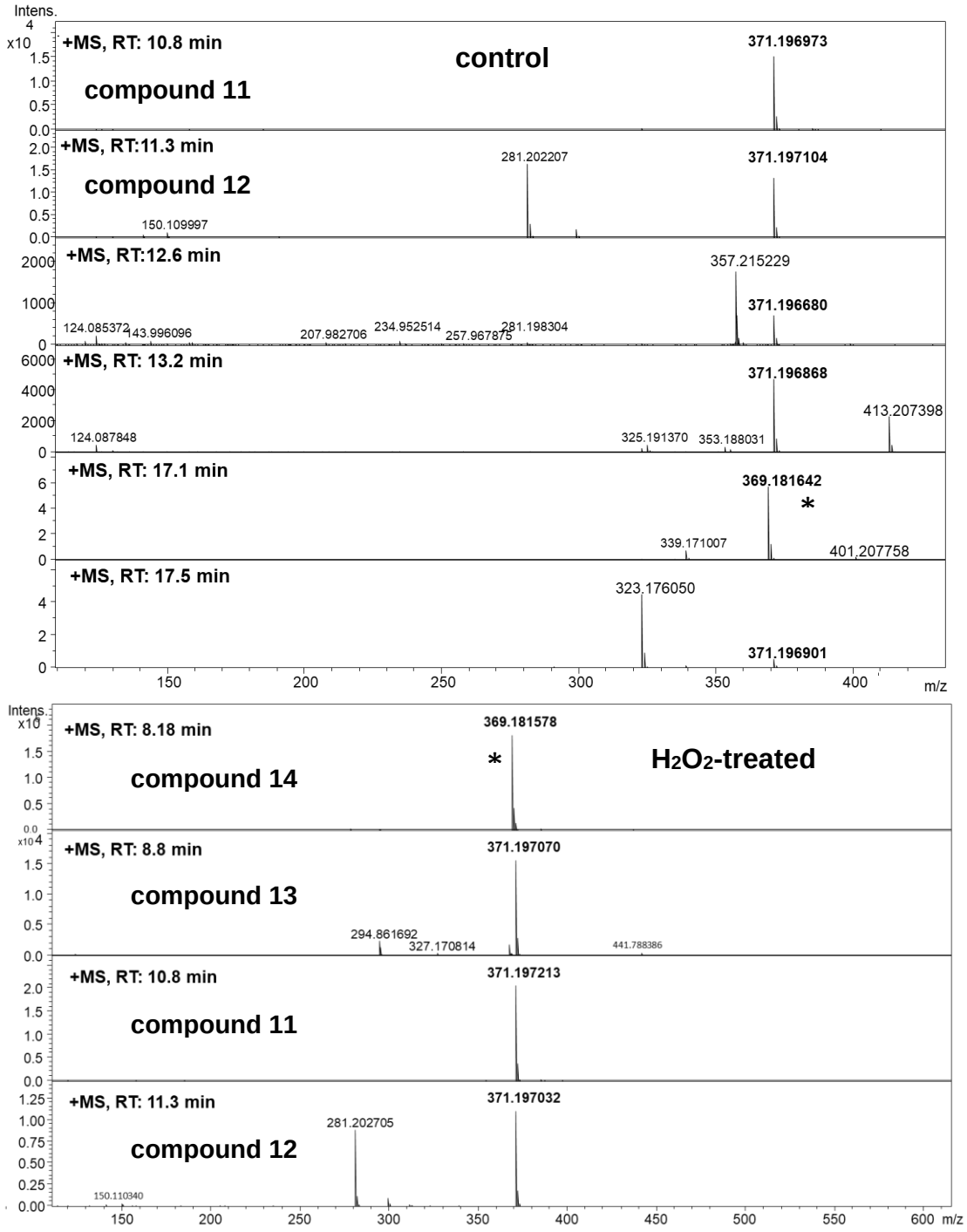


Figure 4- 30: MS-spectra of the peaks with mass (M+H$^+$ = 371*m/z*), in both control, and H_2O_2 - treated plant. * Further analyses revealed that these both compounds reveal a molecular mass of 369 – the positive signal in the EIC (371) is due to the ^{13}C-isotope.

Based on the data mentioned above, it could be concluded that the compounds **11** and **12** (second double peak in the chromatograms of H_2O_2 treated plants and the compound **13** (the second substance of the first double peak) reveal the mass of 371 *m/z* (M+H$^+$), i.e., that of a putative hydroxyvincamine. In contrast, compound **14** (M+H$^+$ = 369 *m/z*) does not represent the postulated intermediate, maybe it corresponds to a further oxidation product (Figure 4-30).

However, based on the previously published data of MIAs isolated before from *V. minor* (Table A-1), apart from a 20-hydroxyvincamine or 19-hydroxyvincamine (depending on the

numbering of secologanin skeleton) also vincatine, which also reveals a molecular weight of 370 is described to occur in *V. minor* leaves (Doepke and Meisel, 1966; Doepke *et al.*, 1969). In order to elucidate the structure of the three compounds (**11**; **12**; **13**), the exact masses of these three compounds were compared with the calculated masses of hydroxyvincamine and vincatine, respectively (Figure 4-31). This comparison clearly outlines that the sum formula of all three compounds is $C_{21}H_{26}N_2O_4$: the calculated deviation of the mass shifts is less than 2ppm for hydroxyvincamine ($C_{21}H_{26}N_2O_4$) but nearly 100ppm for vincatine ($C_{22}H_{30}N_2O_3$). This means that all three substances correspond to a sum formula of $C_{21}H_{26}N_2O_4$ and accordingly, most likely to hydroxyvincamine.

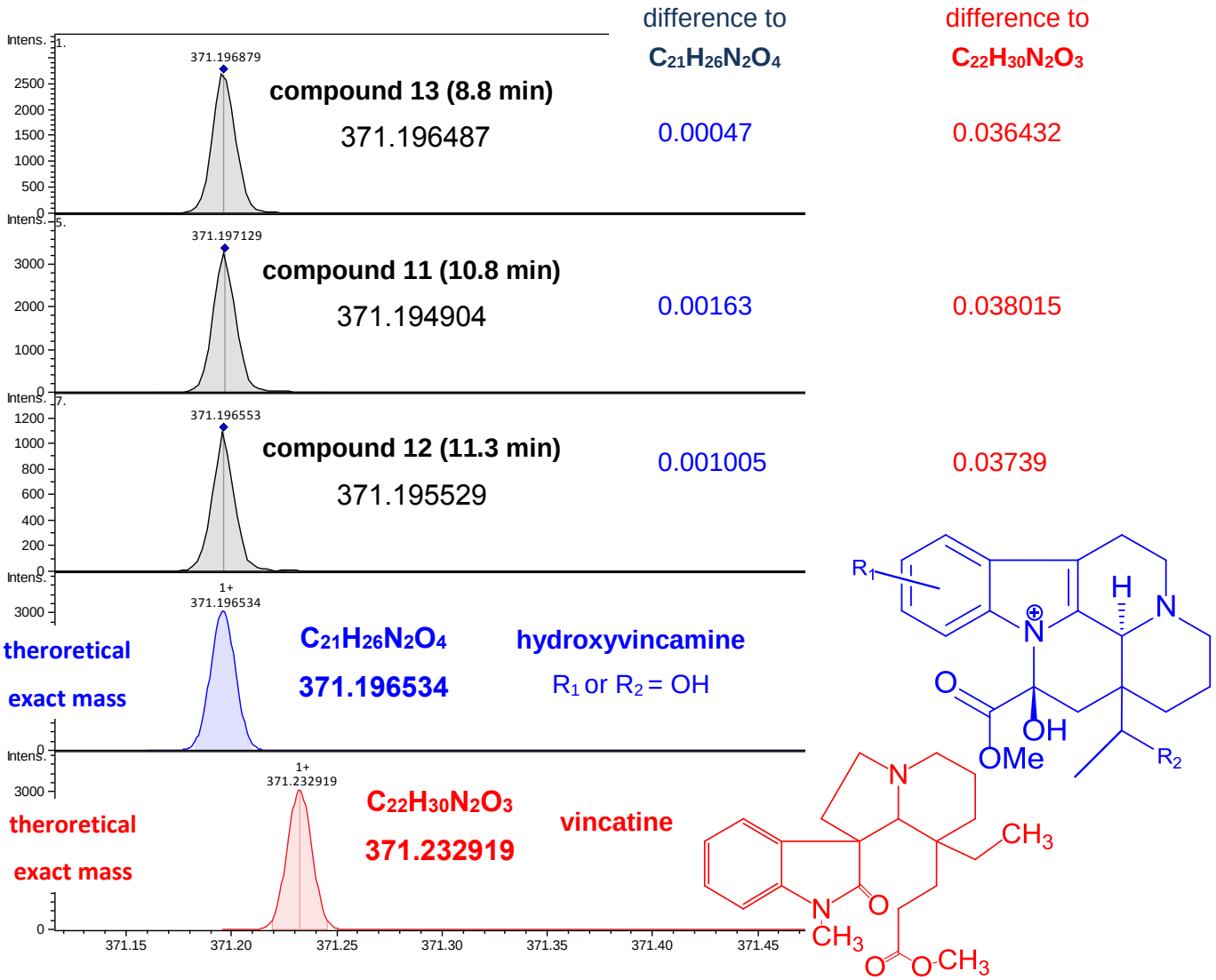


Figure 4- 31: The comparison of molecular weights of estimated values for the two suggested formulas of (hydroxyvincamine and vincatine) and the measured values.

The MS-MS fragmentation patterns of the three compounds, especially those revealing the retention times of 10.8 min (compound **11**) and 11.3 min (compound **12**) have many similarities, but they are also different to some extent, which might be due to different positions of the hydroxyl group (Figure 4-32; 4-33).

The compounds were tentatively further characterized and identified on the basis of the known fragmentation pathway for vincamine to elucidate 9-hydroxyvincamine (**11**) and 20-hydroxyvincamine (**12**), respectively. Both showed the characteristic fragment ions at *m/z* 354.19 due to the losses of OH. The fragment ion at *m/z* 337.1554 and 309.159 is thought to be due to the loss of a further OH followed by that of C_2H_4, respectively, from compound **12** Table 4-13. In addition, compound **12** provided fragment ions at *m/z* 144.0808, whereas compound (**11**) at *m/z* 160.0755 (the 143Da fragment + OH) indicating that the hydroxyl group is attached to indole moiety (Table 4-13; Figure 4-32; 4-33). The 143Da fragment should be generated by the loss of the terpene moiety through C-ring cleavage via Retro Diels Alder (RDA) followed by bond breaking between N_1 and C_{14} (Kumar *et al.*, 2016b).

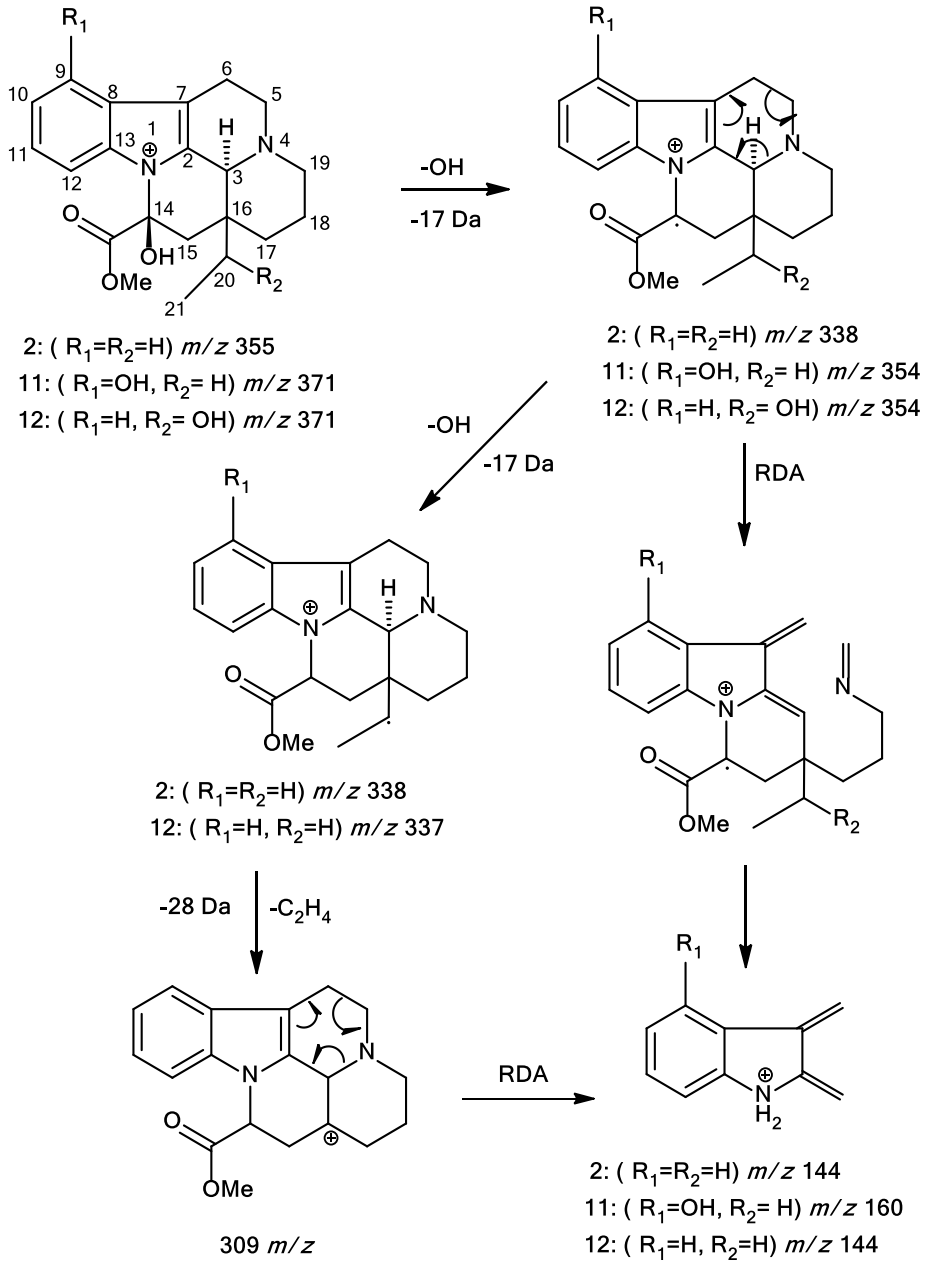


Figure 4- 32: Main fragments of compound 11 and 12 in comparison to those of vincamine (2).

Table 4- 13: Relative abundance (%) of the main fragments of 9-hydroxyvincamine and 20-hydroxyvincamine.

RT min	Compound	$[M+H]^+$	%Relative abundances						
			$[M+1]^+$	$[M-18+1]^+$	$[M-17+1]^+$	$[M-17-17+1]^+$	$[M-17-17-28+1]^+$	*m/z* 144	*m/z* 160
10.8	9-hydroxy-vincamine (**11**)	371.1949	57.8	4.8	100	0	0	0.6	17.3
11.3	20-hydroxy-vincamine (**12**)	371.1955	100	44.2	46.1	14.9	50.1	22.5	0.1

It is worth mentioning that compound **11** showed 50% increase in the concentration in the H_2O_2-stressed plant, whereas no change was detected for compound **12** (Figure 4-29). Whereas the occurrence of 19-hydroyvincamine (**12**) in *V. minor* was already reported earlier (Taylor and Farn-Sworth, 1974), 9-hydroxyvincamine (**11**) was identified for the first time in *V. minor*.

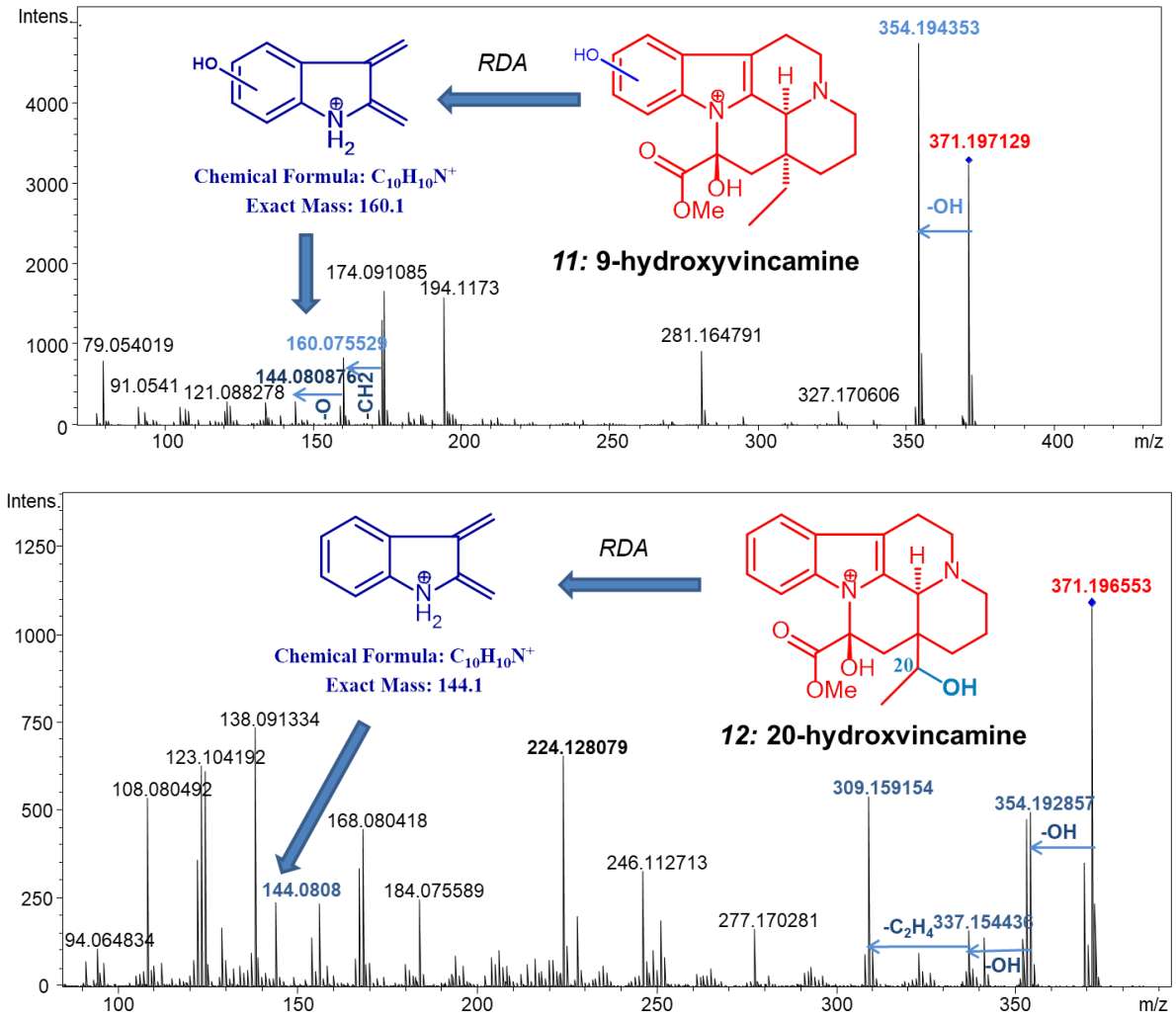


Figure 4- 33: MS-MS spectra of the peaks corresponding to 20-hydroxvincamine and 9-hydroxyvincamine in control and H_2O_2 treated plants. The fragmentation patterns have many similarities, but they are also different to some extent, which might be due to different positions of the hydroxyl group.

Compound 14:

On the basis of its HR-ESI-MS at *m/z* 369.181628, the molecular formula of **14** was determined as $C_{21}H_{25}N_2O_4$ (calculated *m/z* for $[M+H]^+$: 369.180884). The mass 368 m/z of compound **14** might be due to the oxidation of the OH-group of 20-hydroxvincamine, yielding in a corresponding carbonyl derivative (Figure 4-34; the OH-group at C-20 represents the only vicinal hydrogen atom, which could be oxidized to a related carbonyl). The corresponding compound, i.e., 20-oxo-vincamine was already described to occur in *V. minor* and denoted as vincaminine (Trojánek *et al.*, 1961).

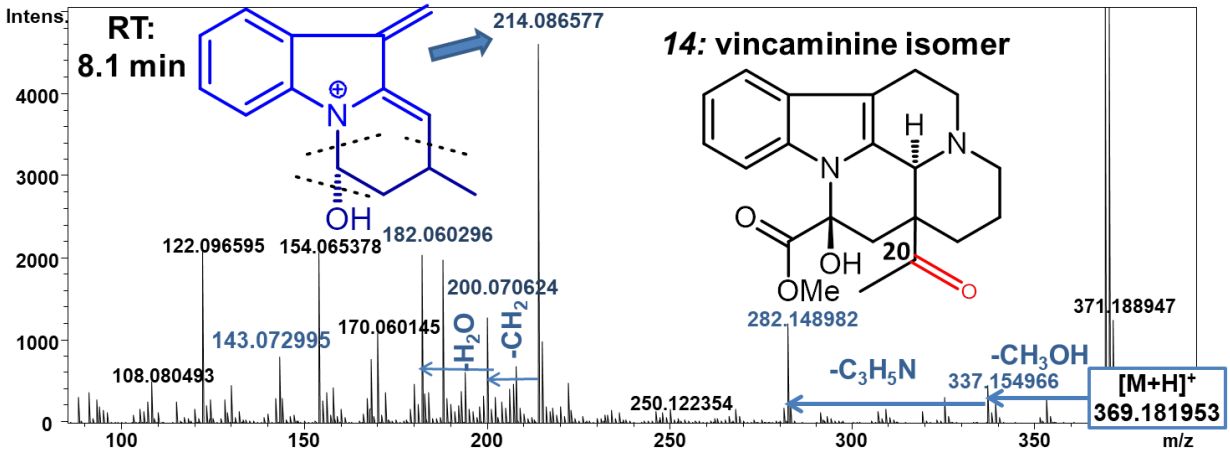


Figure 4- 34: MS-MS spectra of the compounds at 8.1 with mass ($M+H^+$ = 369 *m/z*) in H_2O_2 treated plants.

However, when checking the extracted ion chromatogram (EIC) of all peaks revealing a mass of vincaminine (M+H+ = 369 m/z), it turned out that two further isomers are also present in the stressed plants (Figure 4-35).

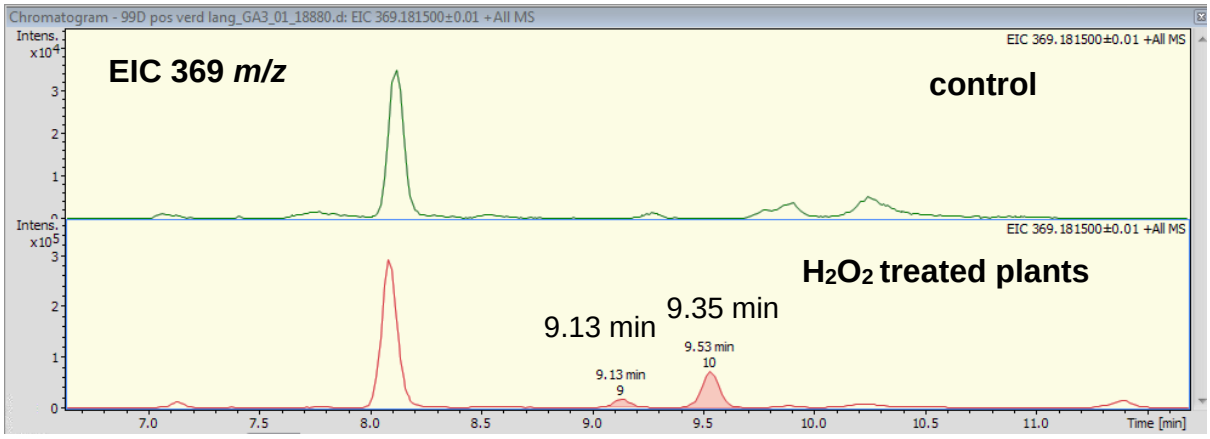


Figure 4- 35: The extracted ion chromatograms (EIC of 396 *m/z*) of control and H_2O_2 treated old leaves. The major unknown alkaloids are denoted 14 isomers.

The MS^2 fragmentations of 20-oxo-vincamine (**14)** are results in a *m/z* 337, putatively due to the loss of methanol. Subsequently, the removal of a C_3H_5N unit is thought to produce a *m/z* 282 (Figure 4-34; Figure 37). In contrast, the MS^2 of the two isomers (RT 9.13 and 9.53 min)

showed a characteristic fragmentation ion at *m/z* 351 which is postulated to result from the loss of water molecule [M – H_2O+H]$^+$ (Figure 4-36; Figure 37). Subsequently, the same fragmentation as for vincamine might occur, i.e., the generation of the characteristic indole moiety at *m/z* 143.0729 or *m/z* 144.080947, respectively (Figure 4-36). Also, in this case, the fragment should be generated by the loss of the terpene moiety through C-ring cleavage via Retro Diels Alder (RDA) followed by bond breaking between N_1 and C_{14} (Figure 4-37; Akhgari *et al.*, 2015; Kumar *et al.*, 2016a). Based on these considerations, it can be stated that compounds at 14 and its isomers indeed are vincaminine.

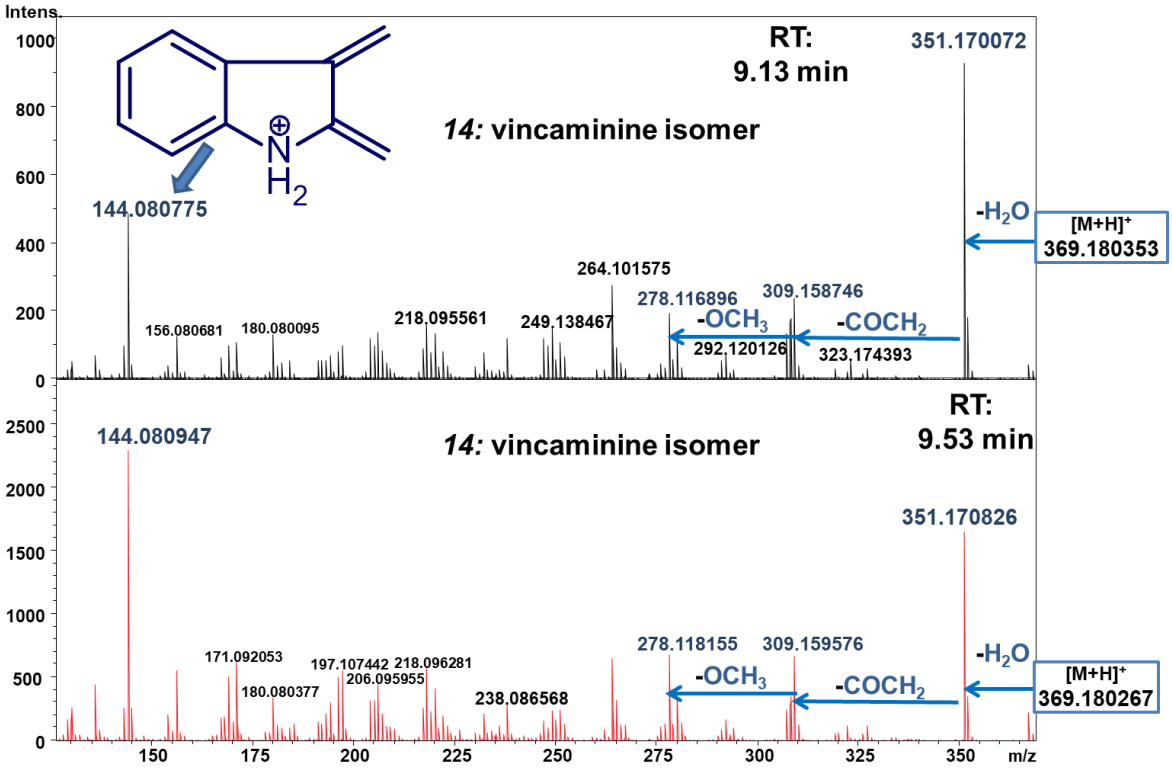


Figure 4- 36: MS-MS spectra of the compounds at 9.13 and 9.35 with mass (M+H$^+$ = 369 *m/z*) in H_2O_2 treated plants. The fragmentation patterns are very similar.

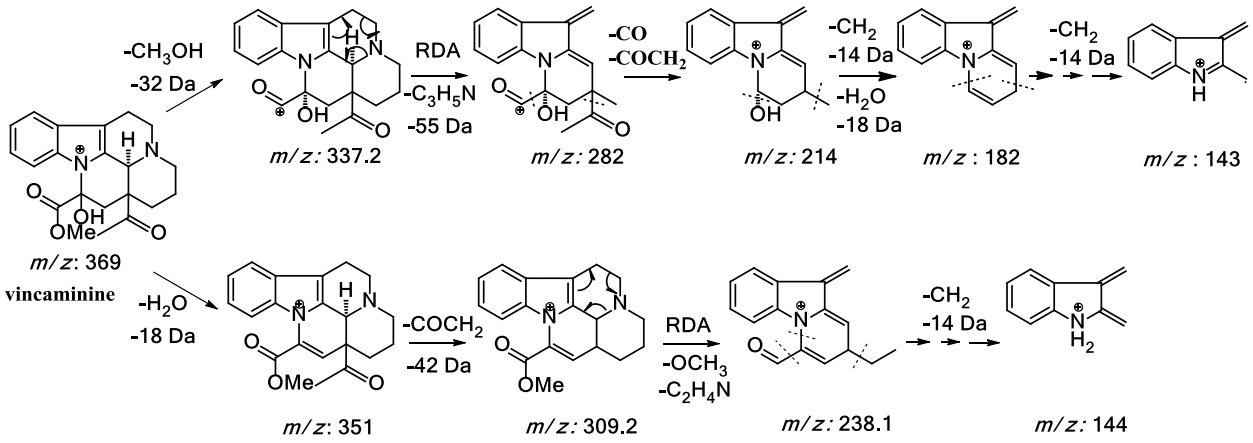


Figure 4- 37: Proposed mass spectral fragmentation of vincaminine (14) and its putative isomers according to MS-MS data.

Compound 13:

On the basis of its HR-ESI-MS at *m/z* 371.196879, the molecular formula of **13** (RT 8.7 min) was determined as $C_{21}H_{26}N_2O_4$ (calculated *m/z* for $[M+H]^+$: 371.196534). The MS^2 spectrum displayed the characteristic fragment ions *m/z* 354 ($[M + H - OH]^+$) and *m/z* 339 ($[M + H - CH_3OH]^+$), which might be due to the loss of a hydroxyl group and methanol, respectively. In addition, the MS^2 fragmentations revealed a base peak fragment at *m/z* 140.107183 ($C_8H_{14}NO$), (Calculated *m/z* for M+H: 140.106990), which indicates that compound **13** might have a hydroxyl or an *N*-oxide piperidine moiety. As the fragmentation patterns of this compound have many similarities to minovincinine (Figure 4-38; (Das *et al.*, 1966)), it is postulated that it corresponds to hydroxyminovincinine.

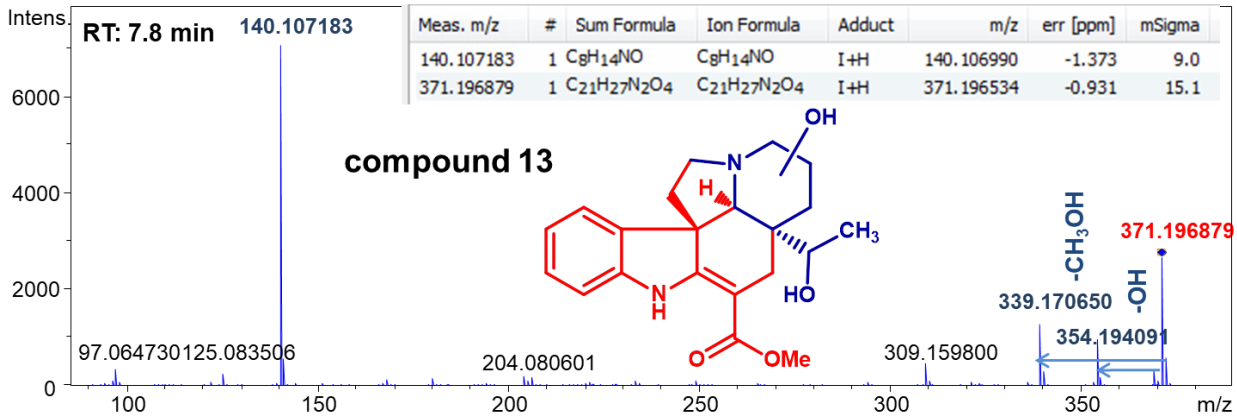


Figure 4- 38: MS-MS spectra of the compound at RT 8.7 min with mass ($M+H^+$ = 371 *m/z*) in H_2O_2 treated plants. The fragmentation patterns have many similarities to minovincinine (figure 4-17).

Further unique compounds detected only on the H_2O_2 treated sample:

As illustrated above, the capability of MS analyses can be massively increased by extracting certain ions from total ion chromatograms (TIC; (Zhou *et al.*, 2008). In the current study, a mass filter was applied to detect further peaks, which are only present or strongly enhanced in their concentration, respectively, in the stressed samples. In this context, the extracted ions from TIC had been the basis to detect isomers, minors, and co-eluting alkaloids. Consequently, many further minor peaks could be detected in H_2O_2 treated samples, which tentatively are identified and displayed in the Table 4-14, and Figure 4-39. Some of these compounds had been identified for the first time to occur in *V. minor*.

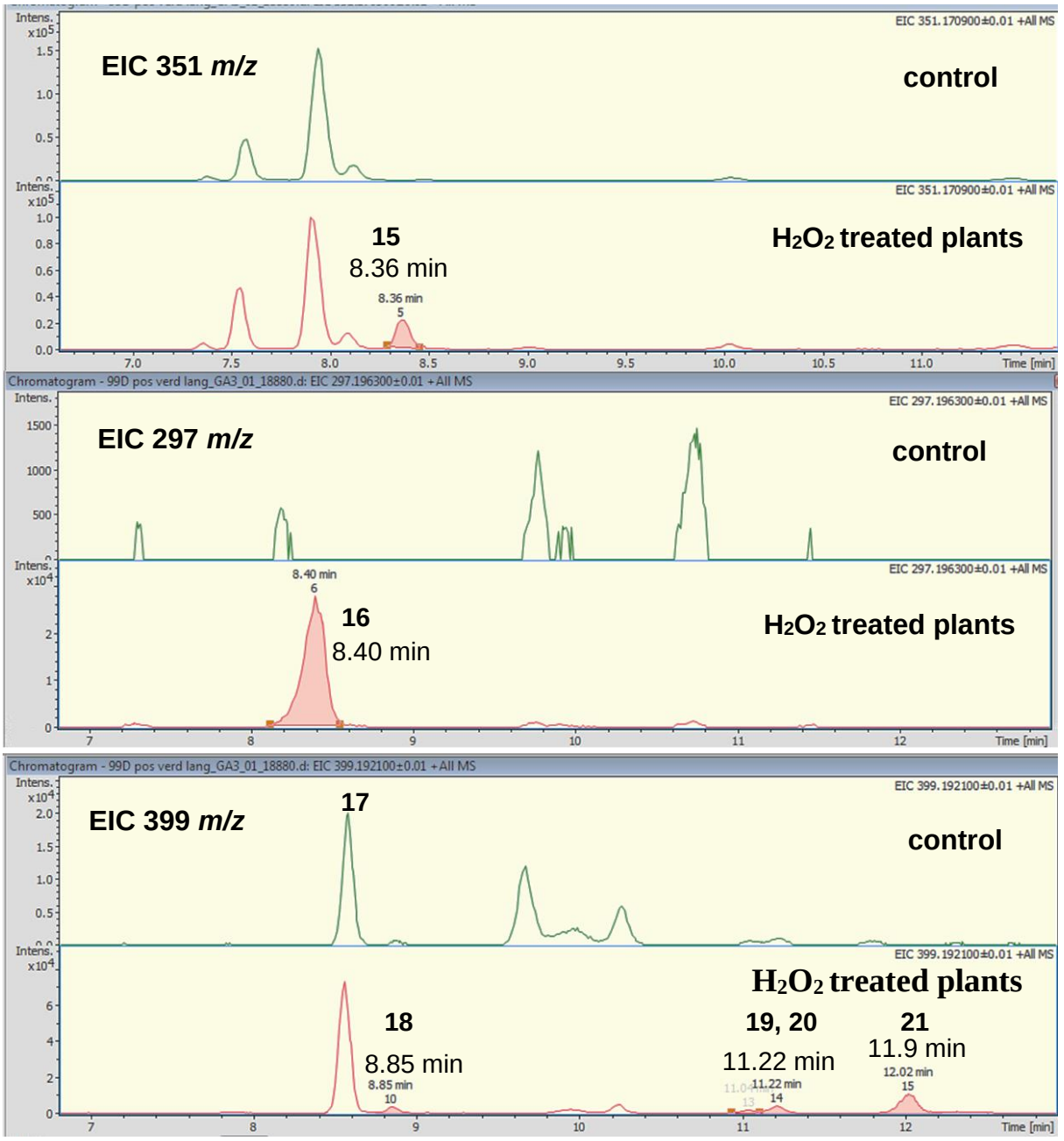


Figure 4- 39: The extracted ion chromatograms (EIC of 351, 297, and 399 *m/z*) of control and H_2O_2 treated old leaves.

Table 4- 14: Identification of further minor compounds from H_2O_2 treated sample by UHPLC-ESI-MS/MS.

≠	RT min	Molecular formula	Obs. mass *m/z*[M+H]$^+$	Calc. mass *m/z*[M+H]$^+$	MS^2 fragment ions *m/z*	Identification	References
15	8.36	$C_{21}H_{22}N_2O_3$	351.1709	351.1703	351 (90), 319 (100), 291, 277, 249	14, 15- dehydro-minovincine	(Langlois and Andriamialisoa, 1979)
16	8.36	$C_{19}H_{24}N_2O$	297.1966	297.1961	297 (100), 279, 253	aspidofractinin-16-ol	(Zhou *et al.*, 2006)
17	8.45	$C_{22}H_{26}N_2O_5$	399.1922	399.1914	399 (100), 367, 355, 244, 232	14,15-didehydro-10-hydroxy-11-methoxy (epi)vincamine isomers	(Zhang *et al.*, 2007)
18	8.85	$C_{22}H_{26}N_2O_5$	399.1917	399.1914	399 (100), 367, 244, 218, 122		
19	11.2	$C_{22}H_{26}N_2O_5$	399.1914	399.1914	399 (100), 381, 216, 122		
20	11.0	$C_{22}H_{26}N_2O_5$	399.1914	399.1914	399 (100), 381, 339, 174	vincinine isomers	(Trojánek *et al.*, 1961)
21	11.9	$C_{22}H_{26}N_2O_5$	399.1918	399.1914	399 (100), 382, 340, 173		
22	18.2	$C_{41}H_{46}N_4O_5$	675.3542	675.3542	675 (100), 114, 108	dimeric alkaloid (unknown)	----------

Compound 15:

On the basis of its HR-ESI-MS (*m/z* 351.170905 [M +H]$^+$), compound 15 (RT 8.36 min) reveals a molecular formula of $C_{21}H_{22}N_2O_3$ (calculated *m/z* for [M +H]$^+$: 351.170319). The MS^2 data of this compound are very similar to those of minovincine ($C_{21}H_{25}N_2O_3$ [M+H] $^+$), except the loss of two hydrogen atoms. As its MS^2 fragmentations were predominated by the loss of methanol yielding in a *m/z* 319.14455 [M – CH_3OH+H]$^+$, compound 15 thought to contain a methyl ester group (Figure 4-40; 4-41). Accordingly, this alkaloid was tentatively identified to be 14, 15- dehydrominovincine (Langlois and Andriamialisoa, 1979), which corresponds a new record for *V. minor*.

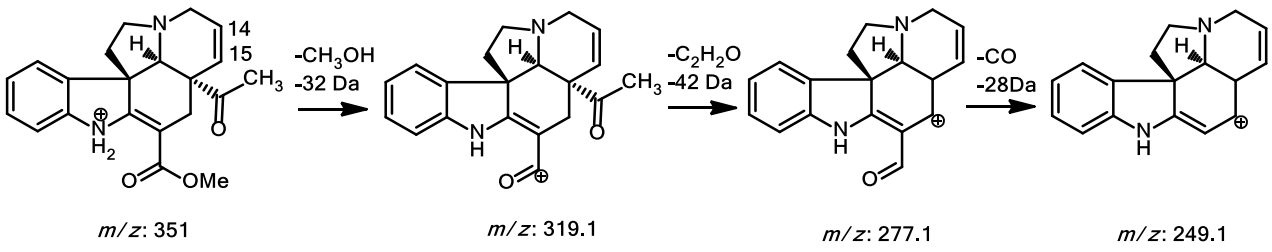


Figure 4- 40: The main fragments of compound 15.

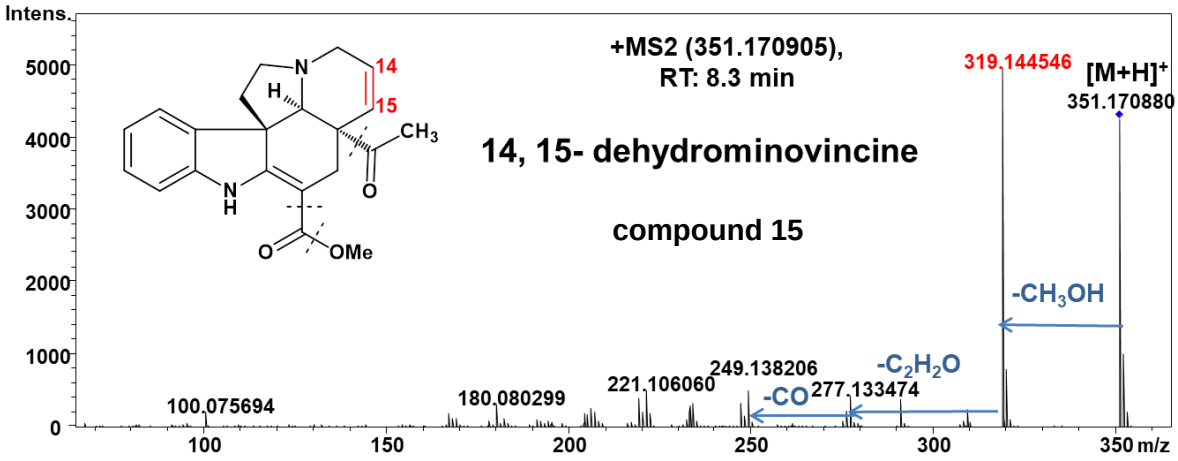


Figure 4- 41: MS-MS spectra of the compound at RT 8.3 min with mass ($M+H^+$ = 351 *m/z*) in H_2O_2 treated plants. The fragmentation patterns have many similarities to minovincine (Figure A- 50).

Compound 16:

On the basis of its HR-ESI-MS (*m/z* 297.196581 $[M+H]^+$), compound **16** (RT 8.36 min) reveal a molecular formula of $C_{19}H_{24}N_2O$ (calculated *m/z* for $[M+H]^+$: 297.196140). The MS^2 fragmentations of **16** (Figure 4-42; 4-43) pointed to the characteristic of loss of water, followed by C_2H_2 unit yielding in *m/z* 279 and *m/z* 253, respectively. The MS^2 spectrum shows high similarities to the previously characterized alkaloid kopsinine (Figure 4-17). These data suggest that this alkaloid corresponds to aspidofractinin-16-ol (Zhou *et al.*, 2006), which putatively was identified for the first time in *V. minor*.

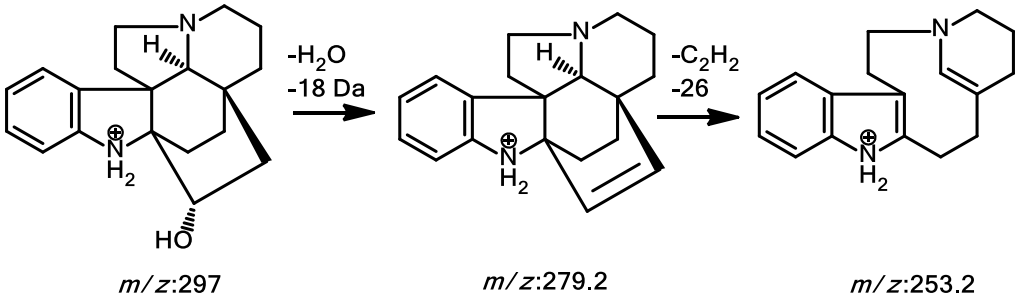


Figure 4- 42: The main fragments of compound 16.

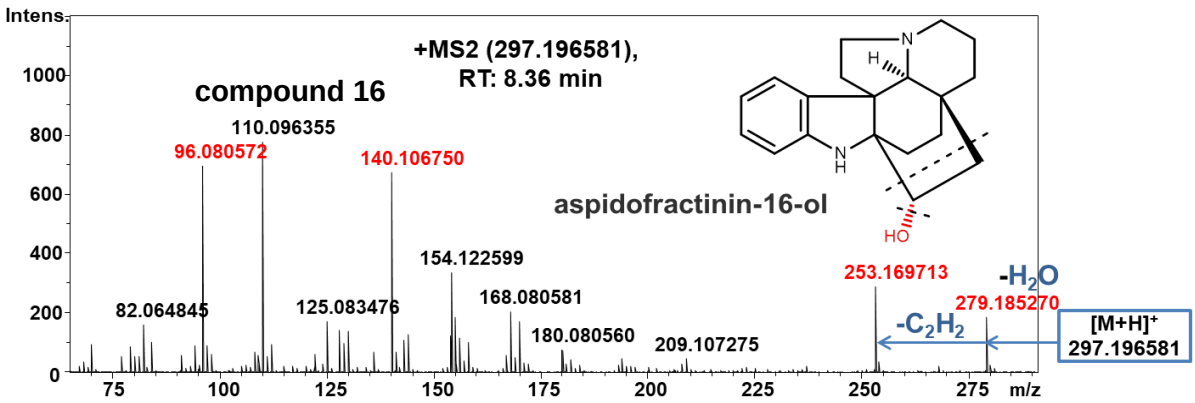


Figure 4- 43: MS-MS spectra of the compound at RT 8.3 min with mass ($M+H^+$ = 297 *m/z*) in H_2O_2 treated plants. The fragmentation patterns have many similarities to kopsinine (**8**) Figure 4-17.

Compound 20 and 21:

On the basis of their HR-ESI-MS (*m/z* 399.191394, and 399.191809 for $[M+H]^+$), the molecular formula of **20 and 21** (RT 11.03 and 11.9 min) were determined for both as $C_{21}H_{26}N_2O_5$ (Calculated *m/z* for $[M+H]^+$: 399.191448). The two compounds reveal a similar MS^2 spectrum, revealing a characteristic ion of *m/z* 381$[M - H_2O+H]^+$, which may result from the loss of water molecule followed by the removal of a $COCH_2$ moiety (42 Da) as outlined for vincaminine (Figure 4-36; 4-44). Subsequently, due to loss of the terpene moiety via *C*-ring cleavage by RDA followed by bond breaking between C_3 and C_{14} (Akhgari *et al.*, 2015; Kumar *et al.*, 2016a), a characteristic ion of indole moiety at *m/z* 174.0915 or *m/z* 173.0832 (143+ OCH_3 (31Da) is generated. Based on these considerations, it could be stated that both compounds are isomers of a methoxylated vincaminine, in which the methoxy group is attached to indole moiety (Figure 4-45). It is very likely that one of these compounds (**20** or **21**) corresponds to 11-methoxyvincaminine, denoted as vincinine, which already had been described to occur in *V. minor* (Trojánek *et al.*, 1961). Accordingly, the other compound (**20** or **21**) should correspond to an isomer of vincinine.

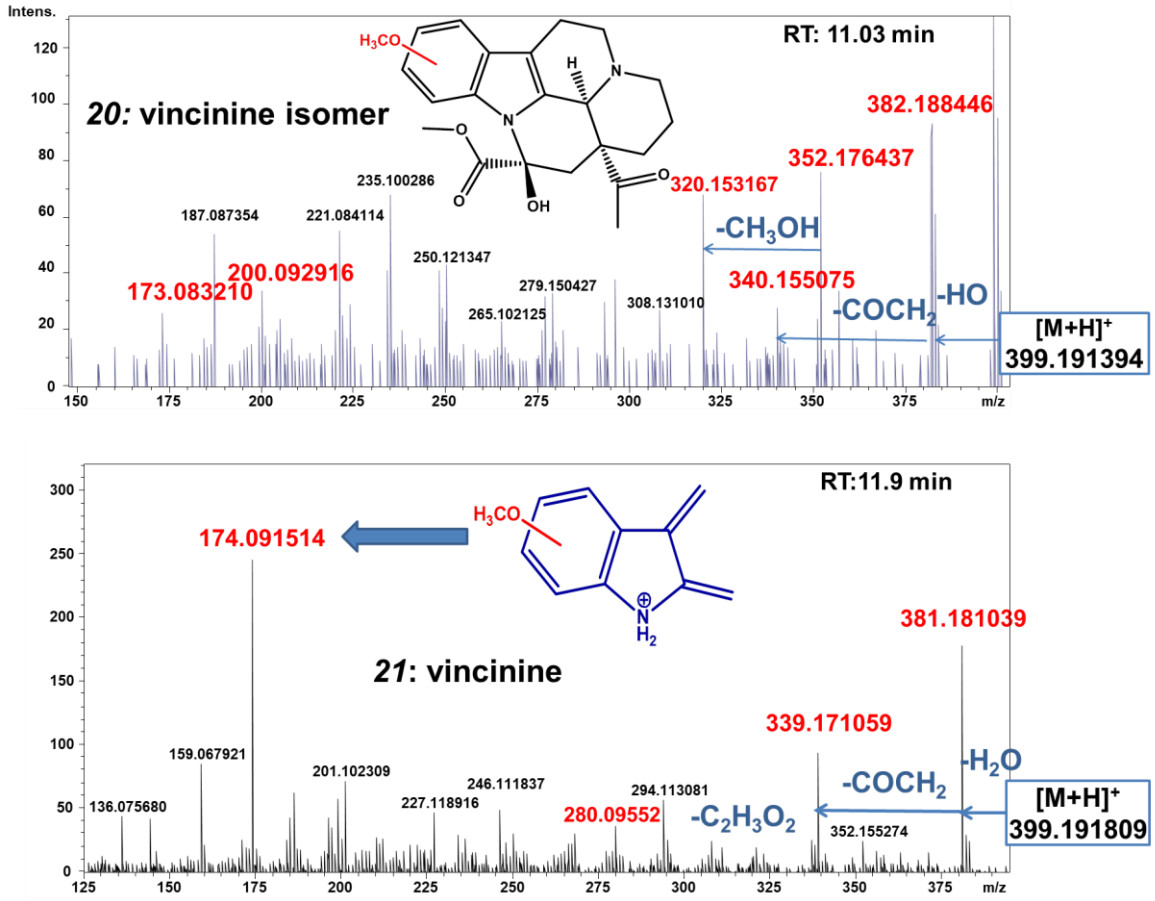


Figure 4- 44: MS-MS spectra of the compounds at RT 11.03, and 11.9 min with mass ($M+H^+$ = 399 *m/z*) in H_2O_2 treated plants. The fragmentation patterns have many similarities to vincaminine isomers (**14**) (figure 4-36).

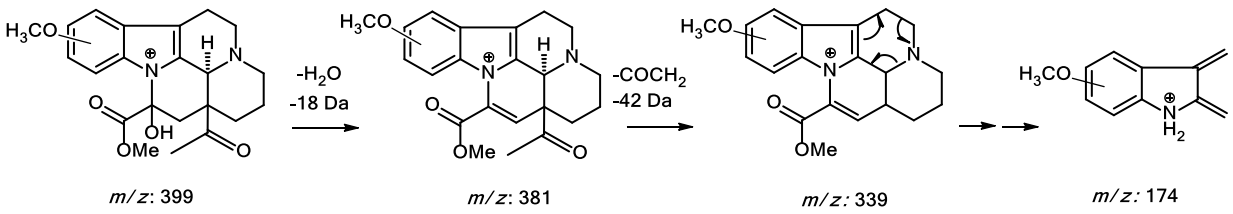


Figure 4- 45: The main fragments of compound 20 and 21.

Compound 17, 18, and 19:

For the compounds **17**, **18** and **19**, a molecular formula of $C_{21}H_{27}N_2O_5$ was estimated on the basis of their HR-ESI-MS (*m/z* 399.1922, 399.191729 and 399.191365 for $[M + H]^+$; the calculated *m/z* for $[M +H]^+$ is 399.191448). The two compounds **17** and **18** (RT 8.45 and 8.85 min) reveal a quite similar MS^2 spectrum that shows a characteristic ion at *m/z* 367, which is thought to result from the loss of methanol $[M – CH_3OH + H]^+$ (Figure 4-46; 4-47). In contrast, compound **19** (RT 11.2 min) showed a characteristic ion at m/z 381$[M – H_2O + H]^+$, which might be due to the loss of a water molecule (Figure 4-46; 4-47). All three compounds exhibit a characteristic ion of an indole moiety at *m/z* 218 or *m/z* 216, which should be due to removal of the terpene moiety via *C*-ring cleavage by RDA followed by bond breaking between C_3 and C_{14} (Akhgari *et al.*, 2015; Kumar *et al.*, 2016a; Kumar *et al.*, 2016b). Based on these considerations, it can be argued that these compounds are similar to vincinine (**20**), however, an additional hydroxyl group is attached to the indole moiety. These compounds could be identified as 17,18-didehydro-10-hydroxy-11-methoxy vincamine isomers, which isolated before from *Ervatamia* species (Zhang *et al.*, 2007).

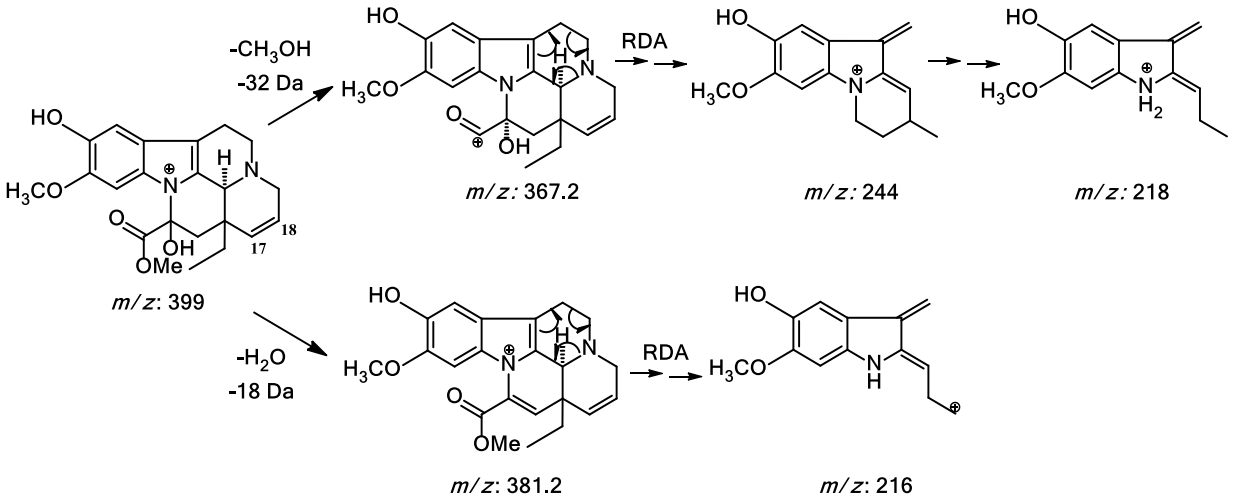


Figure 4- 46: The main fragments of compound 17,18 and 19.

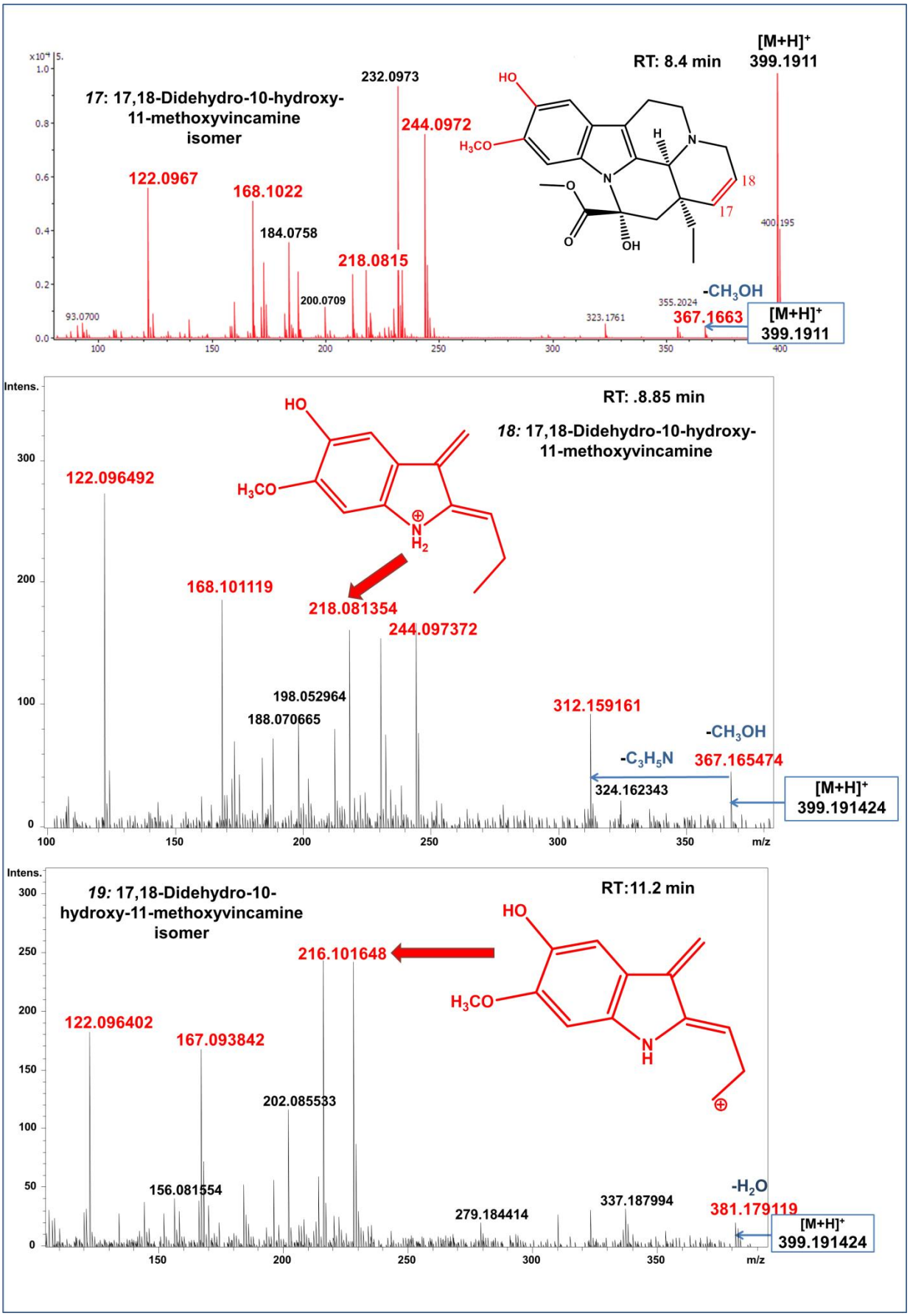


Figure 4- 47: MS-MS spectra of the compounds at RT 8.45, 8.85 and 11.2 min with mass ($M+H^+$ = 399 *m/z*) in H_2O_2 treated plants.

Compound 22:

In addition to the compounds described so far, there is also some evidence for the presence of dimeric indole alkaloids in stressed *V. minor* plants. On the basis of its HR-ESI-MS (*m/z*: 675.3542 for $[M + H]^+$), the molecular formula of $C_{41}H_{46}N_4O_5$ (calculated *m/z* for $[M + H]^+$: 675.3542) was determined for compound **22** (RT 18.3 min), which only was present in the stressed plant. Furthermore, this molecular formula was confirmed by the comparison of the measured and the theoretical isotopic pattern of $C_{41}H_{46}N_4O_5$ (*m/z* 675 +1 and +2; Figure 4-48). Unfortunately, the fragmentation of *m/z* 675 is not very intense for solid structure deductions; however, it generated the typical peak of *m/z* 144 for typical vincamine-type indole alkaloids (Figure 4-49). In addition, when checking the extracted ion chromatogram (EIC) of all peaks revealing a mass from 500 to 900 *m/z*, it turned out that several unique dimeric alkaloids are present only at the stressed plants (Appendix: Figure A- 65).

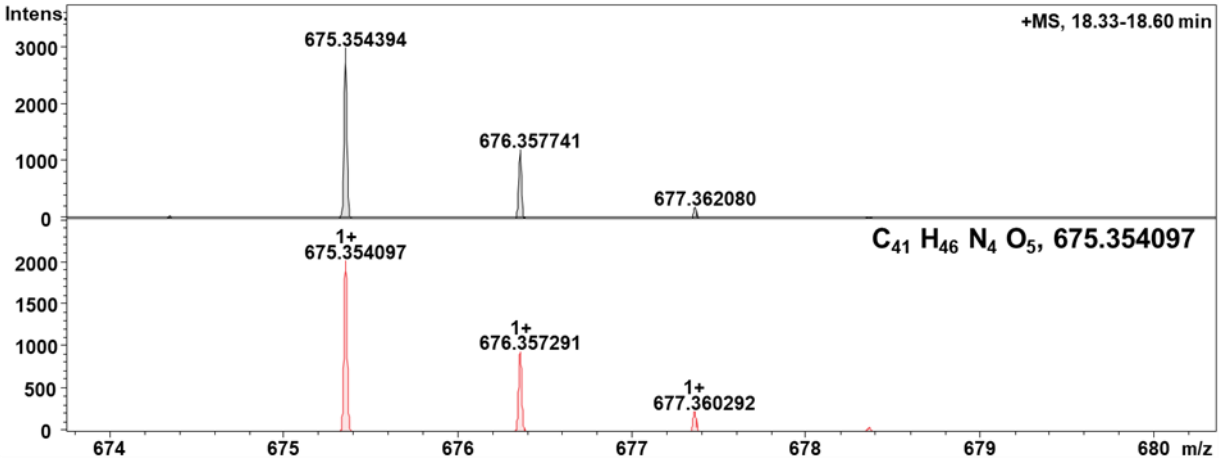


Figure 4- 48: The comparison of the measured isotope pattern of m/z 675 at 18.3min (black) with the theoretical isotopic pattern of $C_{41}H_{46}N_4O_5$ (red).

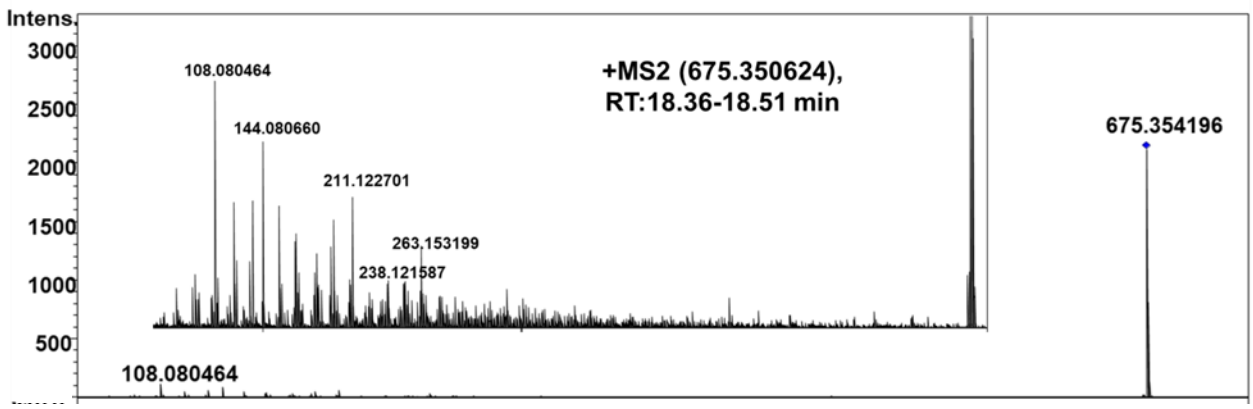


Figure 4- 49: MS-MS spectra of the compounds at RT 18.3 min with mass ($M+H^+$ = 675 m/z) in H_2O_2 treated plants.

Summing-up - 4.4.3 Effect of growth regulators on the composition of alkaloids in V. minor

Whereas the application of SA did not induce major changes in the composition of MIAs, the treatment with MeJA and H_2O_2 significantly impacts on the spectrum.

Similar to MeJA, also the application of H_2O_2 to *V. minor* markedly changed the pattern of the indole alkaloids, and reversed changes in their concentrations have been observed: a decrease in the vincadifformine and vincamine concentrations and a concomitant increase in the minovincine, minovincinine and 9-methoxyvincamine concentration. Whereas in the MeJA treated plants basically 9-methoxyvincamine was produced, in the H_2O_2–treated leaves, also epi-9-methoxyvincamine - putatively derived from epi-vincamine – was present in significant amounts. These findings nicely support the existence of the alterations proposed in Figure 4-18.

A further – but even minor – effect of the H_2O_2 treatment concerns 11-methoxyvincamine and its epimers, i.e., epi-11-methoxyvincamine. Whereas both alkaloids are present in small amounts in the untreated plants, they are lacking in H_2O_2–treated leaves, or are present only in traces.

The extraction of several ions from the TIC revealed clean chromatograms and sharp peaks of compounds of interest, which were used to detect the presence of the putative intermediate in 9-methoxyvincamine, i.e., 9-hydroxyvincamine, and other, minor components, which are tentatively identified for the first time in *V. minor*.

4.4.4 Effect of growth regulators on the alkaloid concentration

As outlined above, the treatment with MeJA strongly alters the alkaloid composition in *V. minor*. In order to elucidate the time frame and the extend of these changes, the experiment was repeated during the blooming time and leaves were collected at four different time intervals. Moreover, also the other growth regulators (H_2O_2, SA) had been applied two times to *V. minor* plants during the blooming phases. Leaves were collected at four different time intervals (1, 4, 8, and 14 days after the treatment) and separated into young and old leaves. The concentration of the different main alkaloids was in control and treated plants are presented in Figure 4-50: 4-52.

The first response after the application of the different growth regulator was the massive increase of vincadifformine in young as well as in old leaves. Especially 4 days after the first application of SA and H_2O_2, (i.e., 24 hours after the second application of the signal molecules) the vincadifformine concentration was strongly increased (Figure 4-51). This enhancement was followed by a drastic decrease, which occurred earlier in the case of SA. This decrease is accompanied by a significant increase in the concentration of vincamine in young leaves. This observation might support that vincadifformine is a putative precursor to vincamine biosynthesis (Wenkert and Wickberg, 1965; Dewick 2009; Kellner *et al.*, 2015; Saxton, 1983a).

Yet, the decrease of vincamine and vincadifformine, which already was outlined above (4.3) reveals a different time pattern in the plants treated with MeJA or with H_2O_2. The most pronounced decrease in vincamine concentration concurred in particular in old leaves with an increase of 9-methoxyvincamine. In contrast, the reduction in vincadifformine concentration and reverse appearance of minovincinine and minovincine was especially observed in young leaves.

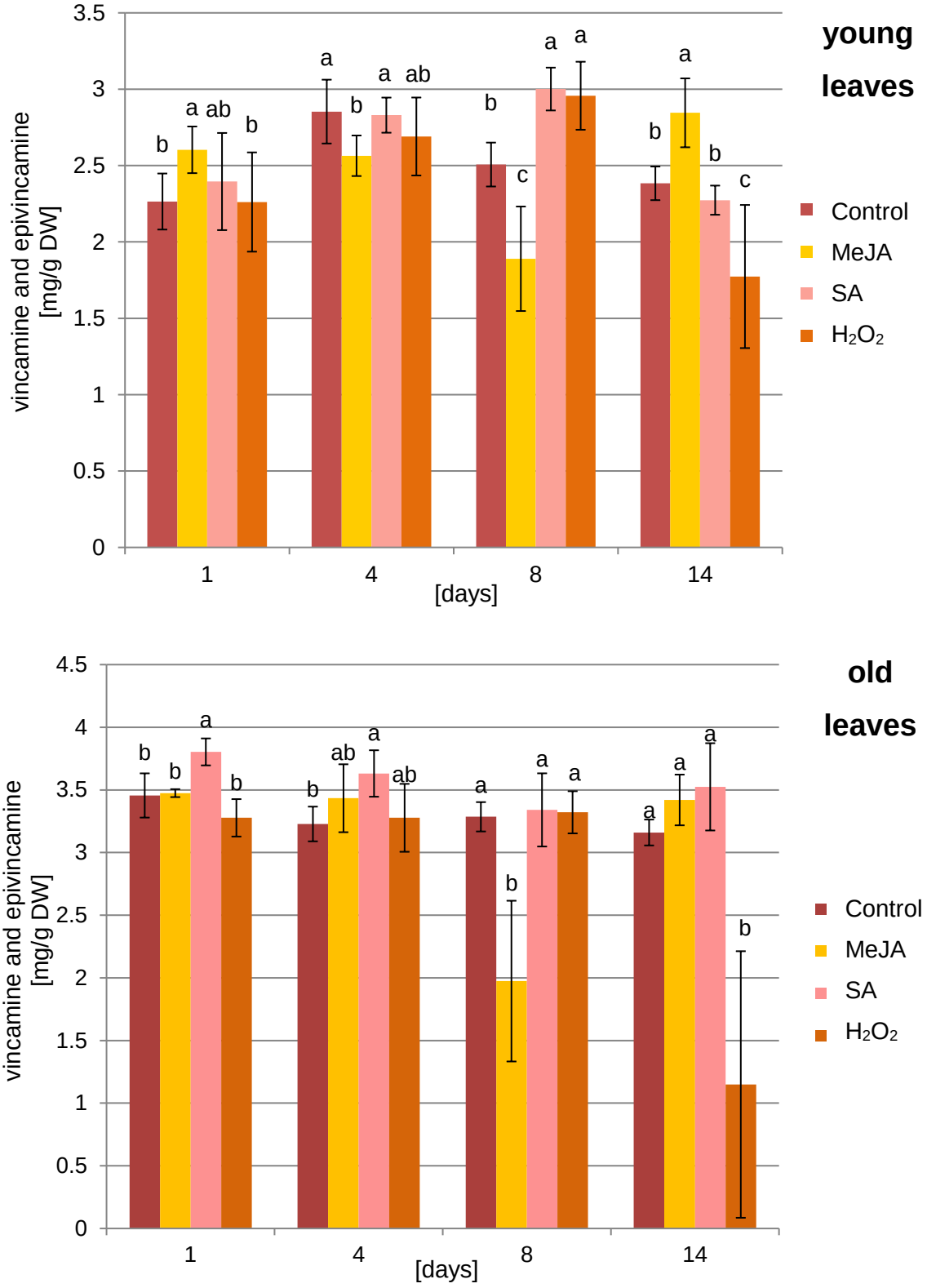


Figure 4- 50: Concentration of vincamine and epivincamine in young and old leaves. Different lower-case letters on top of the column for each period of time (1, 4, 8, 14 days) indicate significant differences (P≤0.05) by the least significant difference (LSD) test. The mean value corresponds to the average value of six independent analyses. Error bars correspond to the standard deviation.

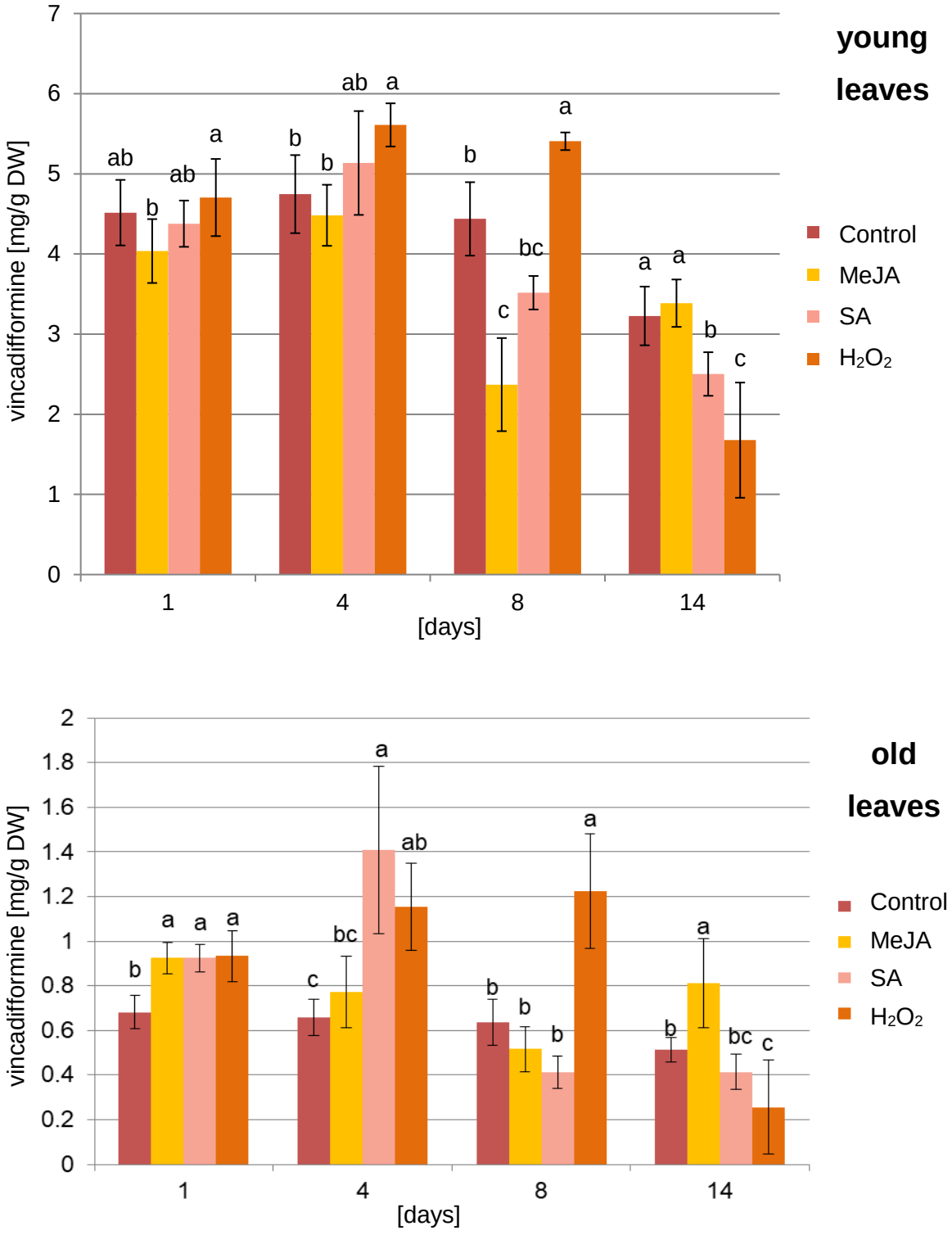


Figure 4- 51: Concentration of vincadifformine in young and old leaves. Different lower-case letters on top of the column for each period of time (1, 4, 8, 14 days) indicate significant differences (P≤0.05) by the least significant difference (LSD) test. The mean value corresponds to the average value of six independent analyses. Error bars correspond to the standard deviation.

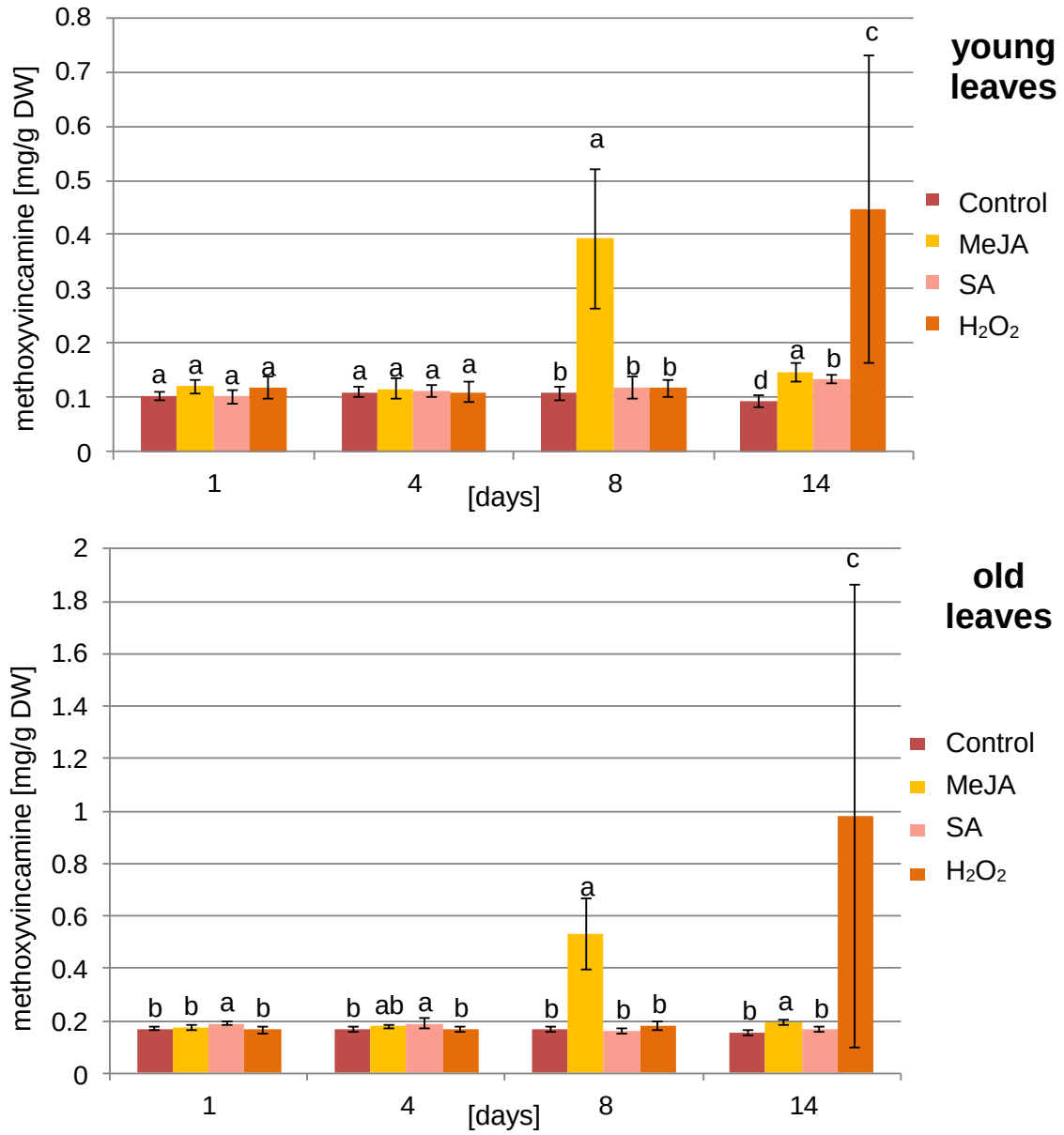


Figure 4- 52: Concentration of methoxyvincamine of young and old leaves. Different lower –case letters on top of the column (for each period of time) indicate significant differences (P≤0.05) by the least significant difference test. The mean value corresponds to the average value of six independent analyses. Error bars correspond to the standard deviation.

These data reveal that the levels and time responses of increase of 9-methoxyvincamine, mino-vincinine and minovincine on the one hand and the decrease of vincamine and vincadifformine on the other hand strongly vary, depending on the growth regulators applied. For better illustration and to recognize the specific effects of a certain treatment, the importance and significant details were extracted from Figure 4-50: 4-52 and displayed appropriately in the following chapters.

Quantitative changes in response to H_2O_2

As outlined, the effects of the different growth regulators vary, especially when focussing on the time frames. In order to get a better overview with respect to the time depending changes, the corresponding data for each growth regulator applied are analyzed and evaluated separately.

H_2O_2 induced a transient increase of the vincamine concentration in young leaves. In old leaves, such increase in the vincamine concentration was not detectable (Figure 4-50a). After two weeks, nearly one-third of vincamine had been disappeared in young and, and even two third in old leaves.

A similar response was detectable for vincadifformine: during the first week after the application of H_2O_2, the alkaloid concentration significantly increased, followed by a massive decrease. Such transient accumulation was observed for young as well as for old leaves. It is worth mentioning that the final decrease of the vincadifformine concentration was very intense. After two weeks, in young as well as in old leaves only half of the vincadifformine accumulated in the controls is present in the stressed plants. When calculating this decline on the basis of the maximal level of vincadifformine – due to the initial increase – this drop off is far more pronounced: after two weeks, nearly two-thirds of the alkaloid had been disappeared in young leaves; and in old leaves hardly 80% had been gone (Figure 4-51a).

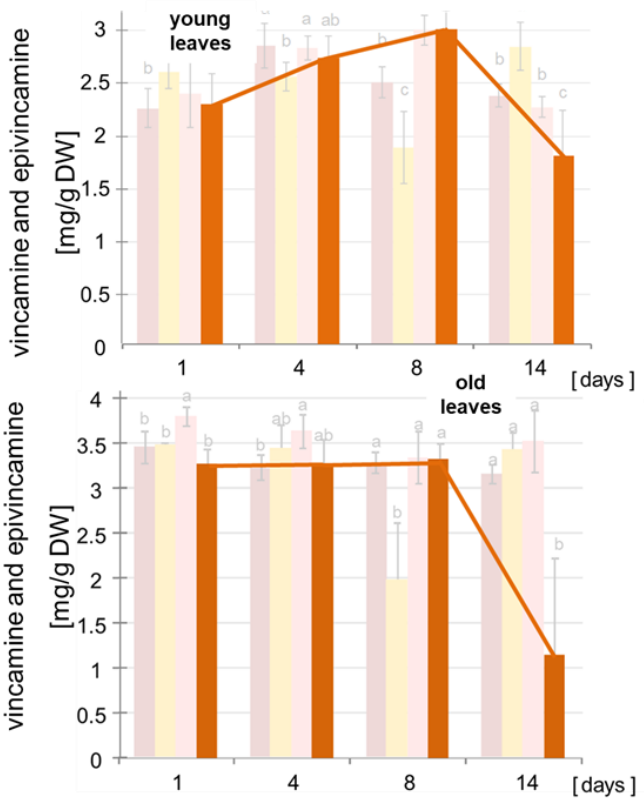


Figure 4- 50a: Changes in concentration of vincamine and epivincamine in young and old leaves in response to H_2O_2 as presented in a Figure 4-50.

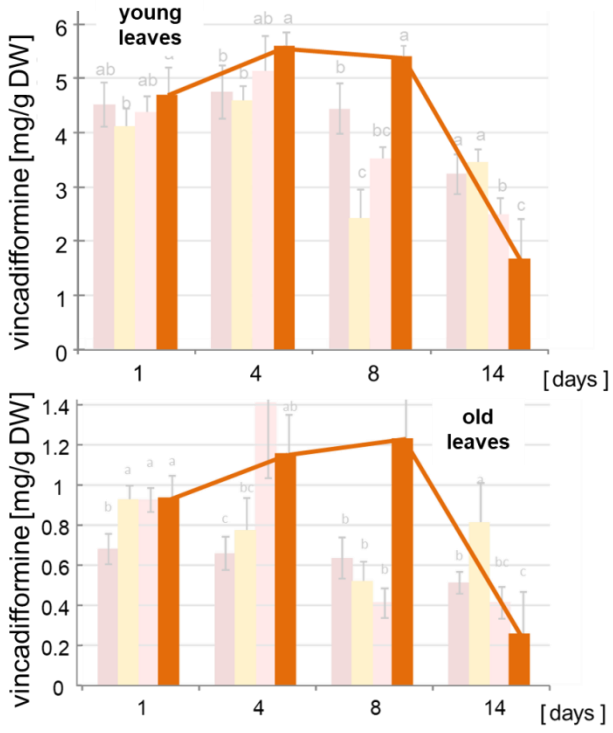


Figure 4-51a: Changes in concentration of vincadifformine in young and old leaves in response to H_2O_2 as presented in a Figure 4-51.

In contrast to vincamine and vincadifformine, the concentration of all other indole alkaloids did not change significantly in response to the H_2O_2, apart from methoxyvincamine, minovincine, and minovincinine.

In the case of methoxyvincamine, which is assumed to be derived from vincamine (Figure 4-18), a significant increase in its concentration was observed in young as well as in old leaves only 14 days after the treatment (Figure 4-52a).

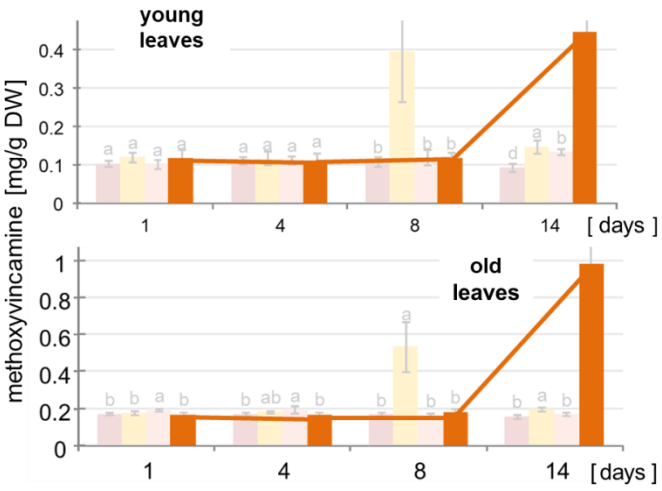


Figure 4-52a: Changes in concentration of methoxyvincamine in young and old leaves in response to H_2O_2 as presented in a Figure 4-52.

This increase exactly coincided with the corresponding decreases of its putative precursor vincamine (Figure 4-50/52a). In this context, it has to be noted that the increase in the concentration of methoxyvincamine in response to the MeJA treatment is also accompanied by a corresponding decrease of vincamine, however, these changes already occurred after 8 days (Figure 4-50a, and Figure 4-50/52a, respectively). The fact that both reverse changes exactly coincided although the effective time of the responses caused by the different growth regulators strongly differed, convincingly supports the biosynthetic pathway mentioned in Figure 4-18.

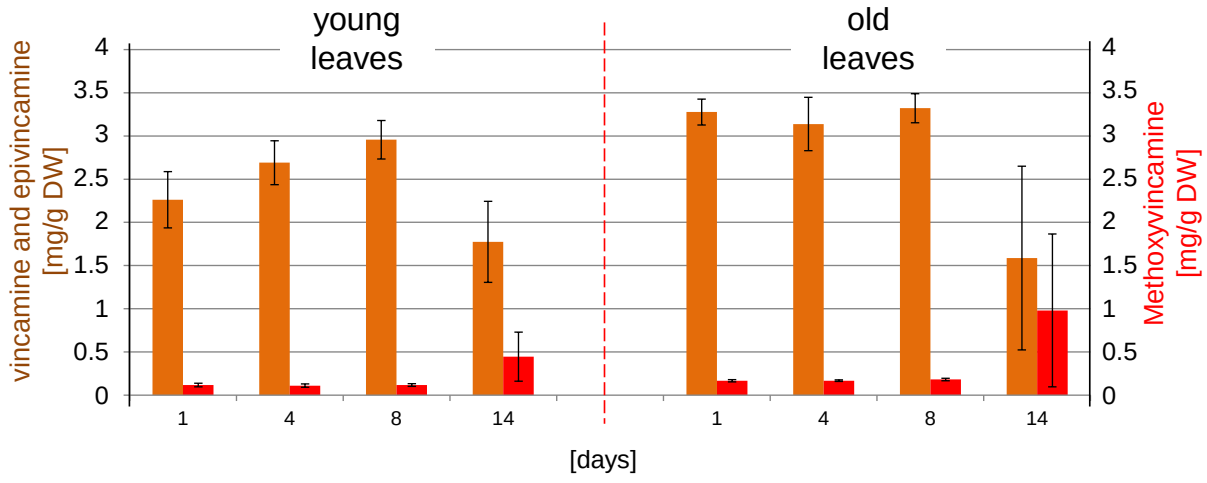


Figure 4-50/52a: The reverse changes in the concentration of vincamine and its putative metabolite –methoxyvincamine- in young and old leaves in response to H_2O_2. The data are extracted from Figure 4-50 and 4-52.

While vincamine and vincadifformine are present also in significant amounts already in unstressed plants, minovincinine and minovincine could only be determined after stress induction. Interestingly, the time frame of the stress responses is equal to that outlined for the generation of methoxyvincamine (Figure 4-53).

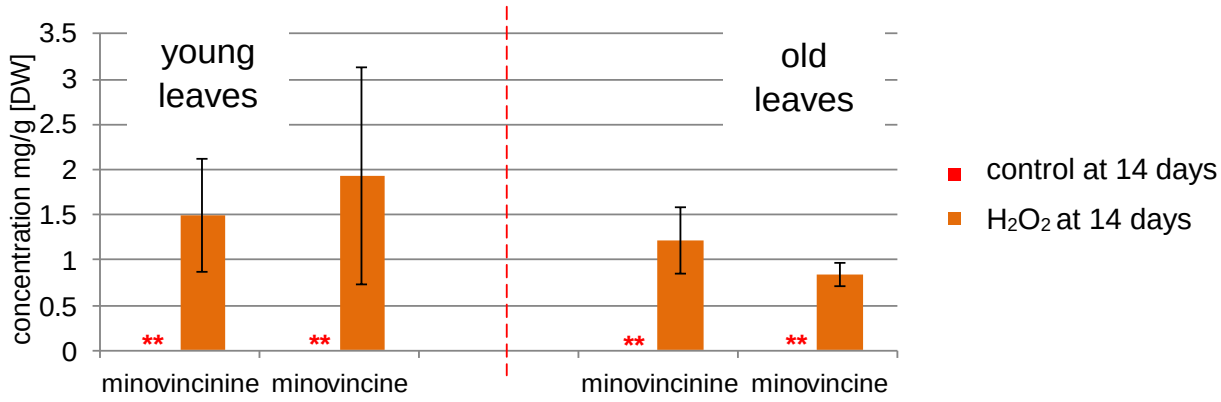


Figure 4- 53: Concentration of minovincinine and minovincine in young and old leaves treated with H_2O_2. ** In the control plants, both alkaloids are below the limit of the detection. Accordingly their concentration was set to be zero. The mean value corresponds to the average value of three independent analyses. Error bars correspond to the standard deviation.

Minovincinine and minovincine, which putatively are derived from vincadifformine, significantly accumulate only 14 days after the H_2O_2 treatment. This increment co-occurs with the decrease of vincadifformine (Figure 51/53a), and thus supports the biosynthetic pathway for minovincinine and minovincine mentioned in Figure 4-18.

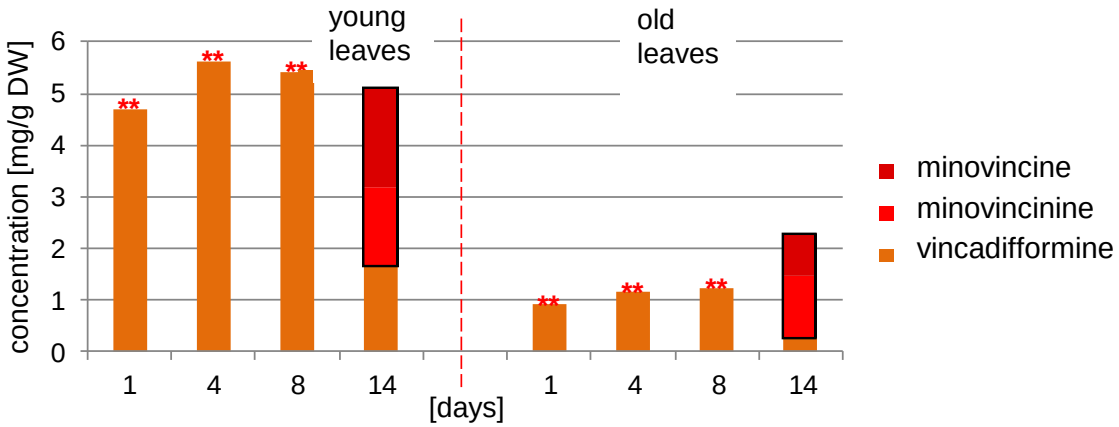


Figure 51/53a: The reverse changes in the concentration of vincadifformine and the putative metabolites minovincinine and minovincine in young and old leaves in response to H_2O_2. When the compounds were below the limit of detection, their concentration is set to zero and mentioned as **. The data are extracted from Figure 4-51 and 4-53.

It is worth mentioning that – in the same manner as outlined for the reverse changes in the vincamine and methoxyvincamine concentration – also the accumulation of minovincinine and minovincine and the corresponding decrease in the vincadifformine content in response to the H_2O_2 treatment occurred retarded compared to the corresponding effects induced by MeJA, which already were detectable 8 to 9 days after the treatment (see below). Yet, also in this both cases, the generation of the putative metabolites coincides with the decline of its precursor, i.e., vincadifformine.

Quantitative changes of indole alkaloids in response to MeJA

As already accomplished for the results related to the application of H_2O_2 also the data from the treatments with MeJA were presented with respect to their time dependency. In comparison to the control, the content of vincamine slightly was increased during the first days. Then, between day 4 and 8, it significantly decreased in young as well as in old leaves (Figure 4-50b). In the course of this decline nearly one-fourth of the alkaloid disappeared in young leaves, and in old leaves, almost half of the vincamine vanished, putatively due to its conversion to methoxy-vincamine (Figure 4-52b).

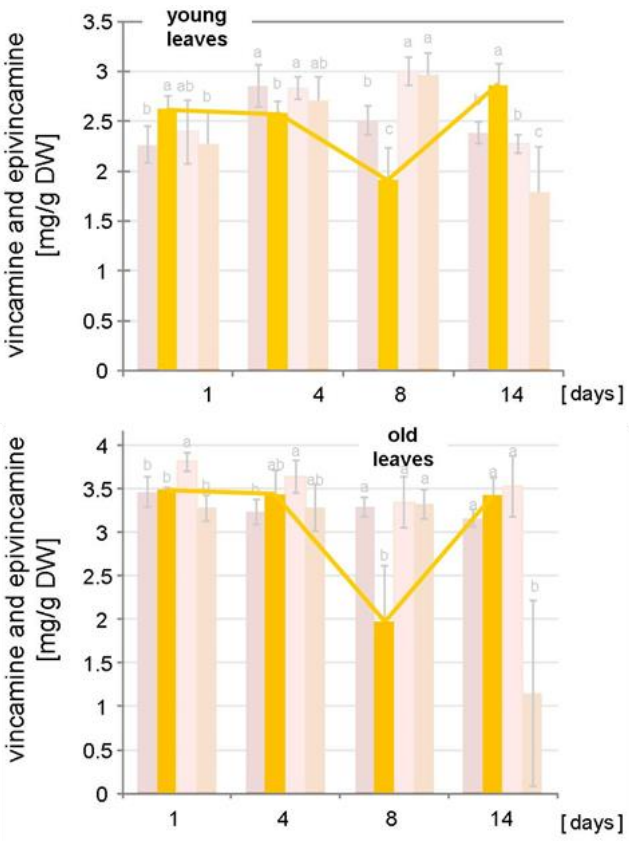


Figure 4-50b: Changes in concentration of vincamine and epivincamine in young and old leaves in response to MeJA as presented in a Figure 4-50.

Subsequently, the vincamine concentration increased again, presumably due to a massive biosynthesis of this alkaloid. When referring this increase to control plants, it corresponds to a 20% higher concentration. Yet, when calculating it on the basis of the minimum level of vincamine present at 8 days, this enhancement is much more pronounced: whilst the second week of after the treatment, the vincamine concentration in increased by about 50% and hardly 70% in young and old leaves, respectively (Figure 4-50b).

Similarly, in old leaves MeJA induced an initial rise of the vincadifformine concentration (about 30%); but such increase was not detectable in young leaves (Figure 4-51b). Subsequently, a

transient decline was observed, resulting in a minimal content of vincadifformine at about 8 days after the treatment in young as well as in old leaves.

When referring these data to the related contents of the control plants, the vincadifformine concentration in the young leaves of the treated plants was only one half, whereas in old leaves the level of treated and control plants is very similar after one week. However, when calculating the extent of this reduction on the basis of the initial high level, almost half of the vincadifformine concentration in old leaves had disappeared, presumably converted to minovincine and minovincinine (Figure 4-54).

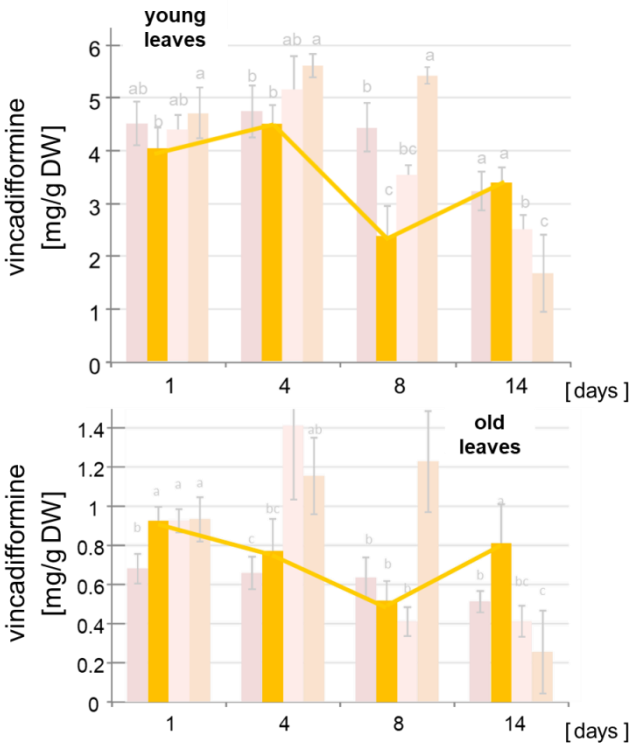


Figure 4-51b: Changes in concentration of vincdifformine in young and old leaves on response to MeJA as presented in a Figure 4-51.

Finally, the concentration of vincadifformine re-increased again, and the highest level was reached two weeks after the treatment. Whereas in old leaves this final level is far higher in stressed leaves than that of the controls, in young leaves no significant difference was detectable. Nevertheless, when calculating the increase in young leaves on the basis of the minimum level of the vincadifformine present at 8 days, the concentration of this alkaloid increased by about 40 %.

The concentration of methoxyvincamine, which is assumed to be derived from vincamine (Figure 4-18), significantly increases in young as well as in old leaves 8 days after the treatment (Figure 4-52b). This increase very exactly coincides with the corresponding decreases of its putative precursor vincamine (Figure 4-50/52b).

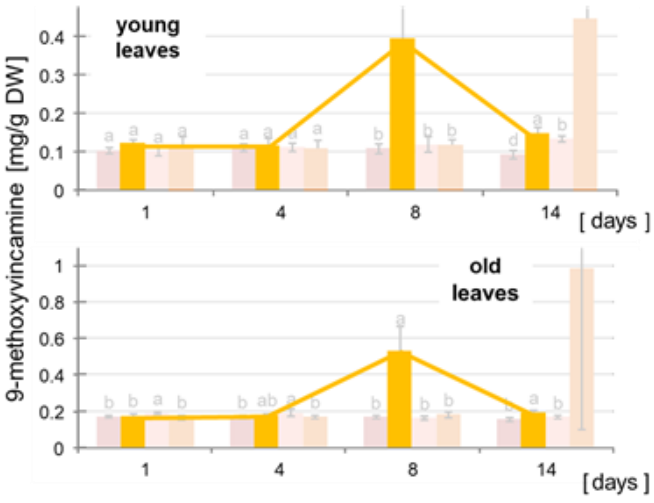


Figure 52b: Changes in concentration of methoxyvincamine in young and old leaves in response to MeJA as presented in a Figure 4-52.

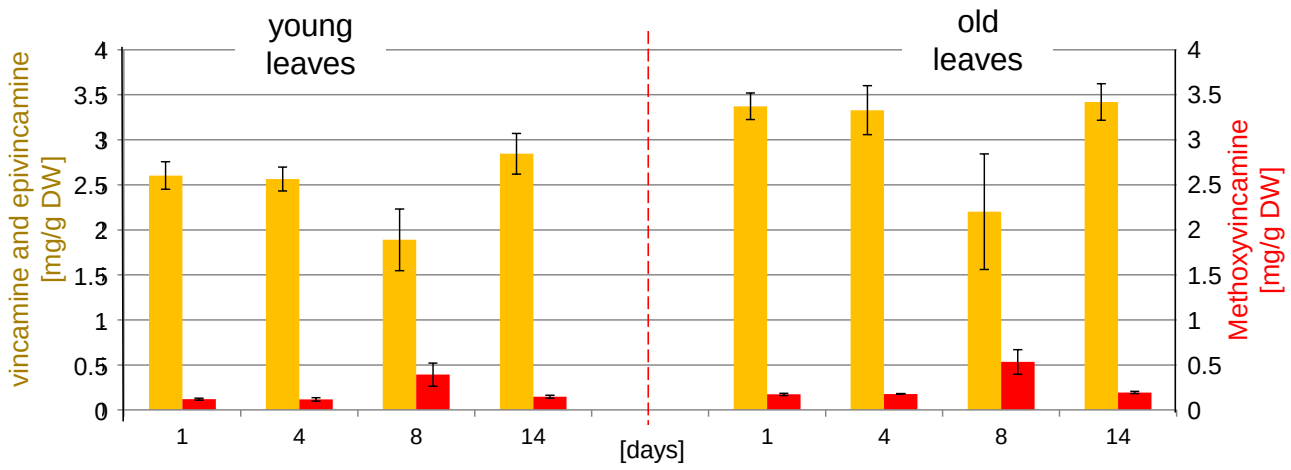


Figure 50/52b: The reverse changes in the concentration of vincamine and its putative metabolite methoxyvincamine in young and old leaves in response to MeJA. The data are extracted from Figure 50 and 52.

Minovincinine and minovincine, which putatively are derived from vincadifformine, significantly increased only after 8 days after the MeJA treatment (Figure 4-51/54b).

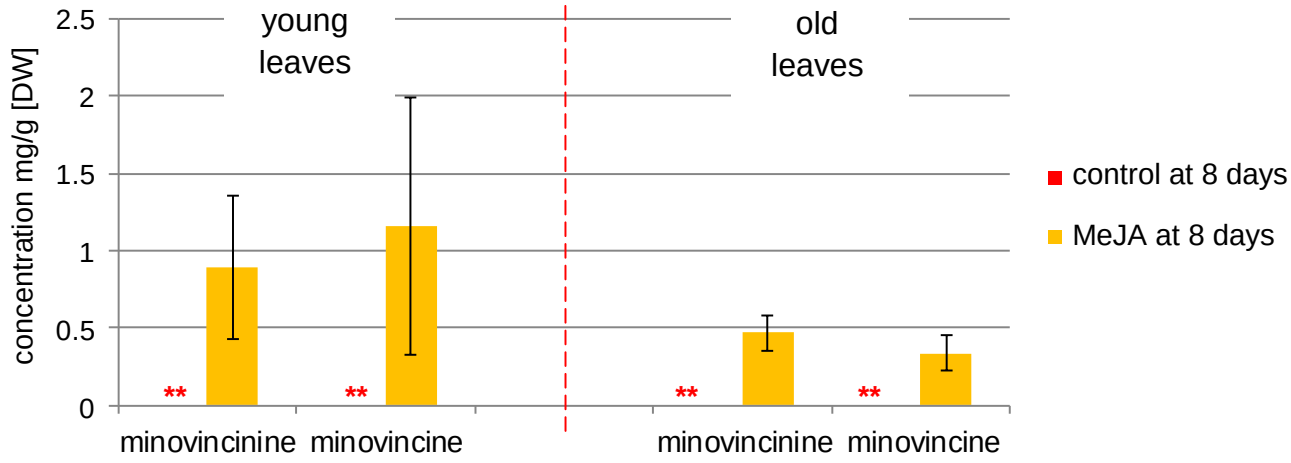


Figure 4- 54: Concentration of minovincinine and minovincine in young and old leaves treated with MeJA. ** In the control plants, both alkaloids are under the limit of the detection; accordingly, their concentration was assumed to be zero. The mean value corresponds to the average value of three independent analyses. Error bars correspond to the standard deviation.

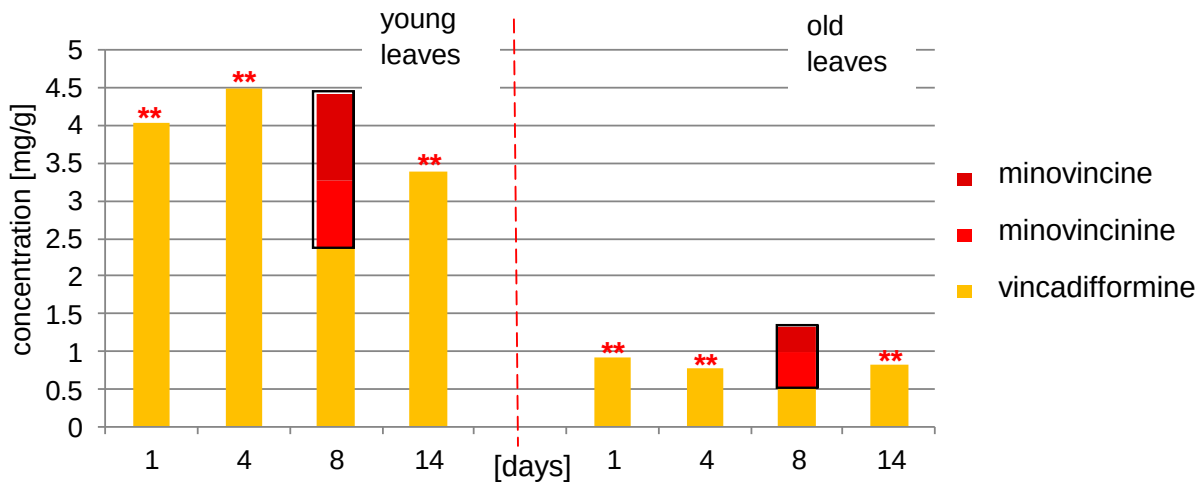


Figure 51/54b: The reverse changes in the concentration of vincadifformine and is putative metabolites minovincinine and minovincine in young and old leaves in response to MeJA. The compounds are under the limit of the detection are set to zero and mentioned as **. The data are extracted from Figure 4-51 and 4-54.

Quantitative changes of indole alkaloids in response to SA

Salicylic acid (SA) is another important signaling molecule in plants. It has been well documented that SA is involved in the defense responses related to the systemic acquired resistance (SAR) by activating certain defense-related genes (Thomma *et al.*, 2001; Hasanuzzaman *et al.*, 2017). Previous studies have shown that exogenous addition of SA induced indole alkaloid production in *C. roseus* within a very short time period (Pan *et al.*, 2010). In contrast, the application of SA to suspension cultures and seedling had no effect on the concentration of indole alkaloids in *C. roseus* (Aerts *et al.*, 1996; Guo *et al.*, 2013). Other authors reported the opposite effect: when SA is applied to seedlings or suspension cultures, respectively, the content of alkaloid increased (Godoy-Hernández and Loyola-Vargas, 1997; El-Sayed and Verpoorte, 2004). Accordingly, also SA was applied to *V. minor* plants in this study.

The treatment of *V. minor* with SA during the blooming time resulted in a transient increase of the vincamine and vincadifformine accumulation in the leaves. Yet, in old leaves, this increase of vincamine concentration is only visible when referred to the controls (Figure 4-50c).

In contrast to the relatively slight changes in the vincamine content (Figure 4-50c), the SA treatment caused massive changes in that of vincadifformine, i.e., 4 days after SA applications, in old leaves, its concentration is nearly doubled in comparison to the untreated controls. But then, it strongly is reduced again. After about one week, the vincadifformine concentration is even lower than that of the controls. In young leaves, initial increase, as well as the subsequent decrease, is far less intense (Figure 4-51c).

It is noteworthy to mention that – in contrast to the treatment with MeJA and H_2O_2 – apart from one unique sample (Appendix: Figure A-66) – neither methoxyvincamine nor minovincine and minovincinine had been produced in response to the SA treatment.

Obviously, SA does not induce a conversion of vincamine to methoxyvincamine and of vinca-difformine to minovincine and minovincinine, respectively.

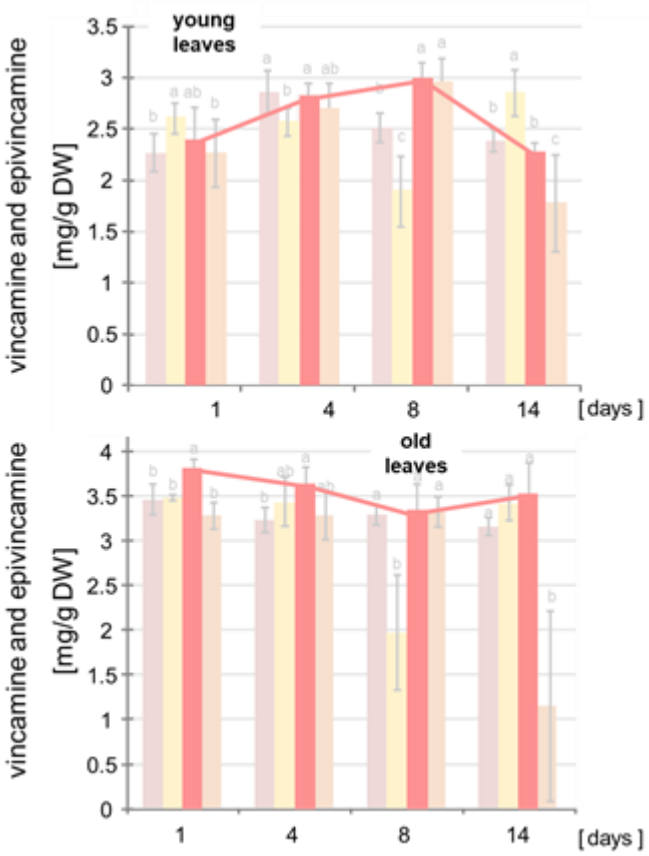


Figure 4-50c: Changes in concentration of vincamine and epivincamine in young and old leaves in response to SA as presented in a Figure 4-50.

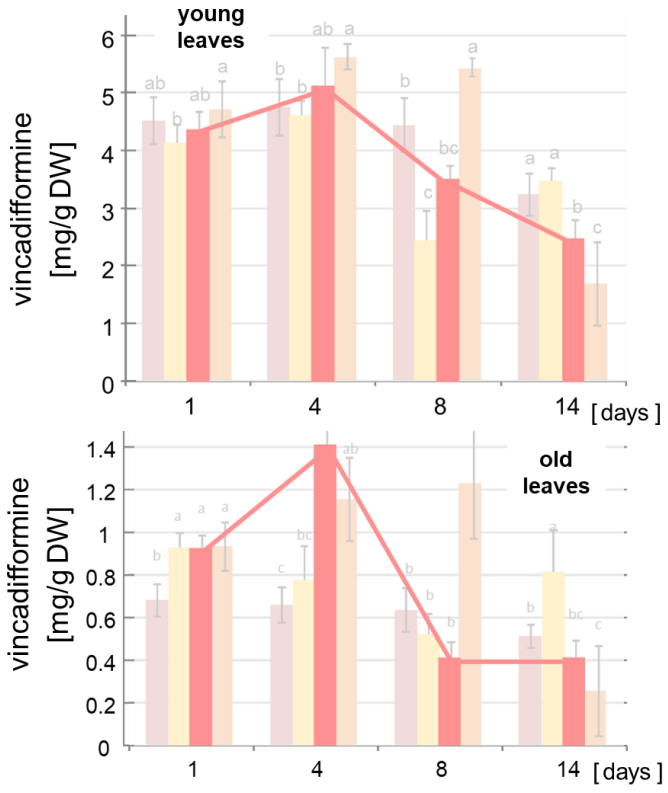


Figure 4-51c: Changes in concentration of vinca-difformine in young and old leaves on response to SA as presented in a Figure 4-51.

4.4.5 Effect of inhibitors on the MeJA induced conversion of indole alkaloids

As outlined in the scientific background (section 2.3.4.2), various inhibitors of oxidative enzymes are described, which mainly act on enzymes that are induced by the jasmonic acid induction pathway (Morgan and Shanks, 1999; Guo *et al.*, 2011). In this manner, the application of naproxen changed the alkaloid composition of *C. roseus* suspension culture (Guo *et al.*, 2011), and the combination of naproxen with H_2O_2 increases the total alkaloid content of *V. minor* hairy root culture (Verma *et al.*, 2014b) . Accordingly, also naproxen was applied to *V. minor* plants in this study.

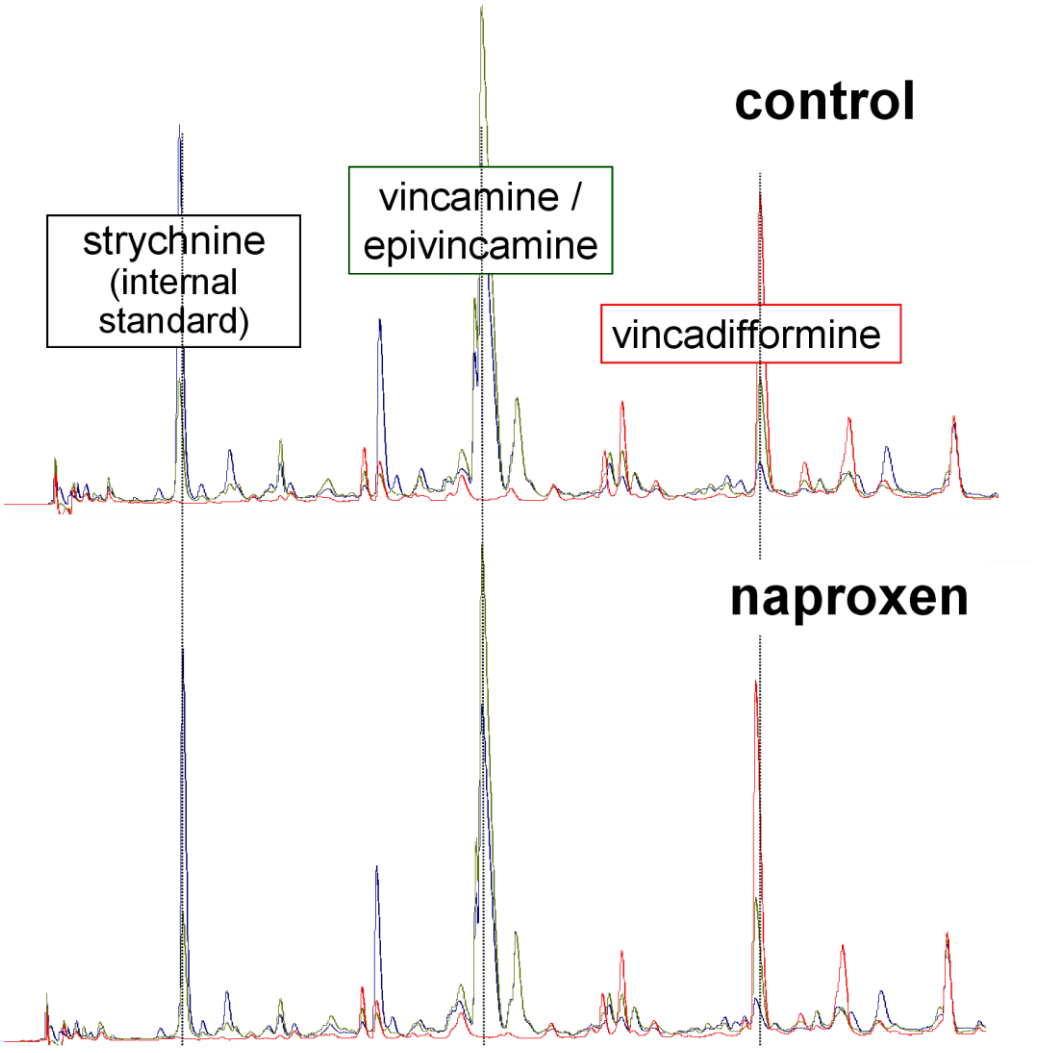


Figure 4- 55: Impact of naproxen on the alkaloid spectrum of *V. minor*. The plants were sprayed with naproxen two times during the experiment and collected at 9 days.

These results suggest that naproxen primarily has no pronounced effect on the composition and concentration of the indole alkaloids present in *V. minor* plants (Figure 4-55). In principle, this outcome could have been predicted, since only those oxidative enzymes, which putatively are induced by MeJA, should be inhibited by naproxen. In the case of the indole alkaloids of *V. minor*, these are the enzymes involved in the MeJA induced conversion of vincamine to 9-

methoxyvinamine, and of vincadifformine to minovincine and minovincinine, respectively (Figure 4-18). Consequently, naproxen has to be applied to plants, in which the corresponding hydroxylation steps, i.e., the conversion of vincadifformine and vincamine are implemented by MeJA. Accordingly, in a further experimental approach naproxen was sprayed together with MeJA (Figure 4-56).

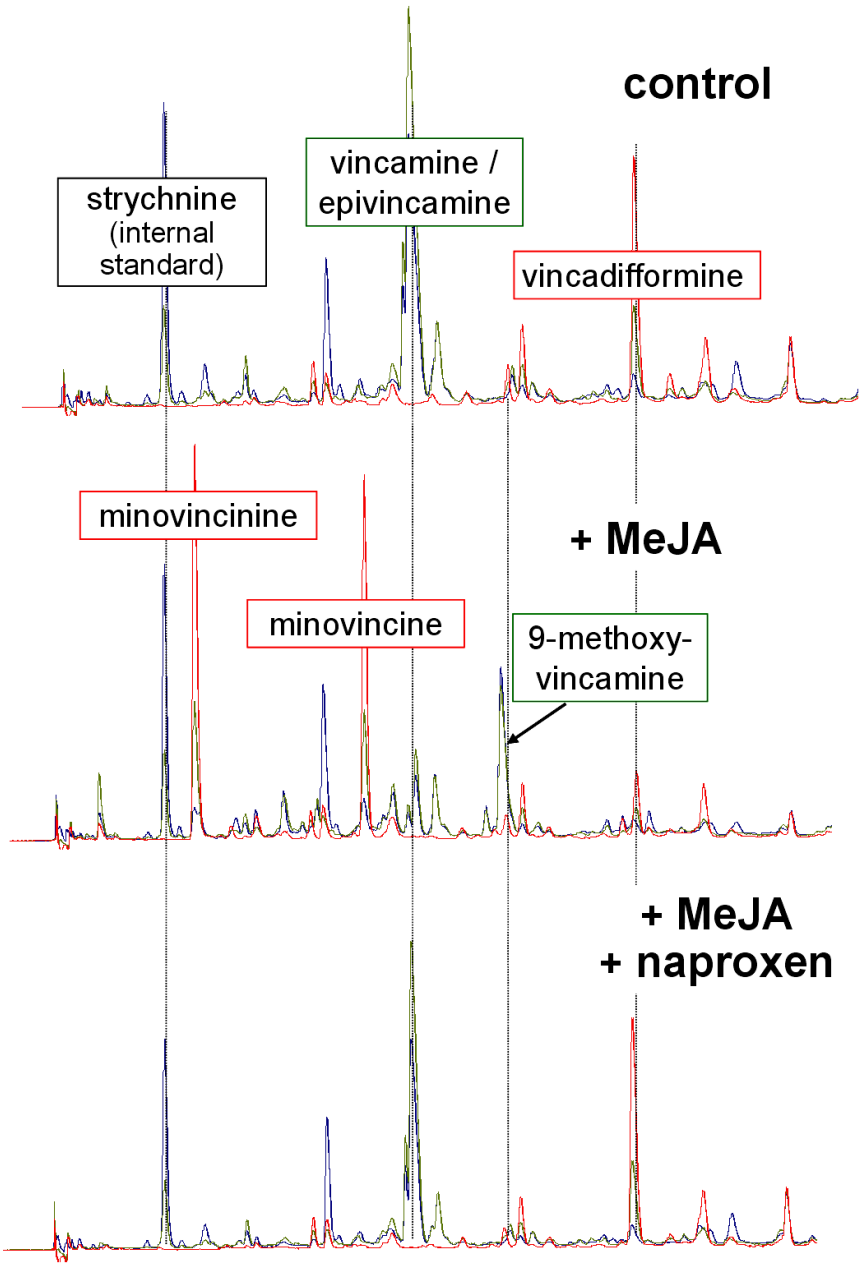


Figure 4- 56: Impact of the concomitant application of naproxen and MeJA on the alkaloid spectrum *V. minor*. The plants were sprayed two times during the experiment and collected at 9 days.

As predicted, the MeJA induced changes in the alkaloid pattern of *V. minor* could be suppressed by the inhibition of the oxidative enzymes involved by naproxen. It has to be mentioned that the inhibition of the MeJA induced conversion of vincamine to 9-methoxyvinamine, and of vincadifformine to minovincine and minovincinine, respectively (Figure 4-56). This

suppression is very efficient and only traces of the transformation products had been produced after three days (Figure A-69), and completely inhibited at 9 days (Figure 4-56). From this, it could be deduced, that naproxen very efficiently inhibits the corresponding cytochrome P450 enzymes in *V. minor*. This feature might help to identify the corresponding enzymes. Yet, we have to consider that the outcome of this experiment could also be caused by another mechanism involved. It could not be excluded that naproxen – in contrast to a direct inhibition of the enzymes – also might act indirectly, e.g., by suppressing the transduction of the MeJA signal. The related coherences are discussed in detail in chapter 5 (Discussion 5.2).

In addition of naproxen, also, resveratrol which is also known to inhibit corresponding P450 enzymes (Chan and Delucchi, 2000; Fan and Mattheis, 2001; Laden and Porter, 2001; Piver *et al.*, 2001; Kutil *et al.*, 2014), was applied to *V. minor* plants to modulate the alkaloid biosynthesis in *V. minor*. Yet, based on the knowledge that resveratrol is taken up from the soil and translocated into the leaves (Tucker Serniak, 2016; Radwan (2017), pers. Communication; and a corresponding experiment was done for barley plants (Appendix; Figure A-67)), in a first approach, resveratrol was added 4 times to *V. minor* via the irrigation water.

Analogously to the experiments with the application of naproxen, also in the case of resveratrol, no major changes in the alkaloid pattern had been observed, i.e., no conversion of vincadiffor-mine to minovincine or vincamine to is induced. Accordingly, in a further experiment, MeJA and resveratrol had been applied concomitantly.

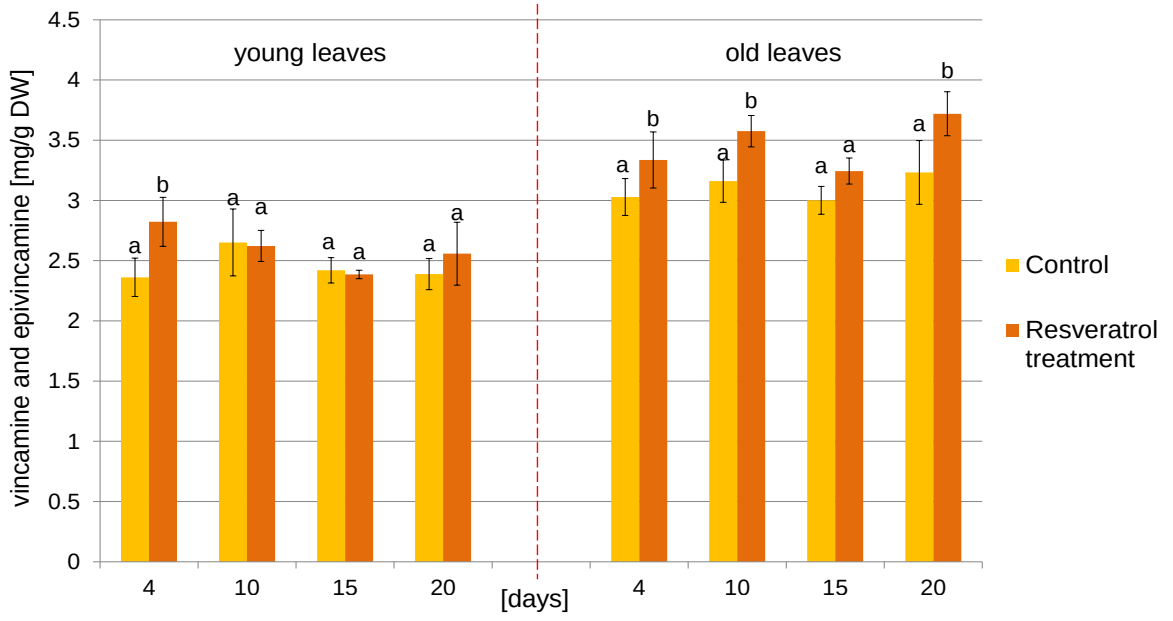


Figure 4- 57: Concentration of vincamine in young and old leaves. Different lower –case letters on top of the column for each period of time (1, 4, 8, 14 days) indicate significant differences (P≤0.05) by the least significant difference (LSD) test. The mean value corresponds to the average value of six independent analyses. Error bars correspond to the standard deviation. Vincamine corresponds to the sum of vincamine and epivincamine.

Treatment of *V. minor* with resveratrol resulted only in minor differences in the vincamine content (Figure 4-57). Whereas no clear trend could be estimated in the young leaves, in the older ones, the application of resveratrol in all cases resulted in a slightly higher concentration of vincamine (Figure 4-57). This means that vincamine is not massively converted to 9-methoxyvinamine. This is underlined by the finding, that the latter one is present in the resveratrol-treated plants in the same amount as in the controls (see Appendix; Figure A-68).

In the same manner, resveratrol had no major effect on the concentration of vincadifformine (Figure 4-58) and a conversion to neither minovincinine nor minovincine could be excluded. This is confirmed by the finding that both of the putative conversion products are absent in the resveratrol-treated plants (see Appendix; Figure A-68).

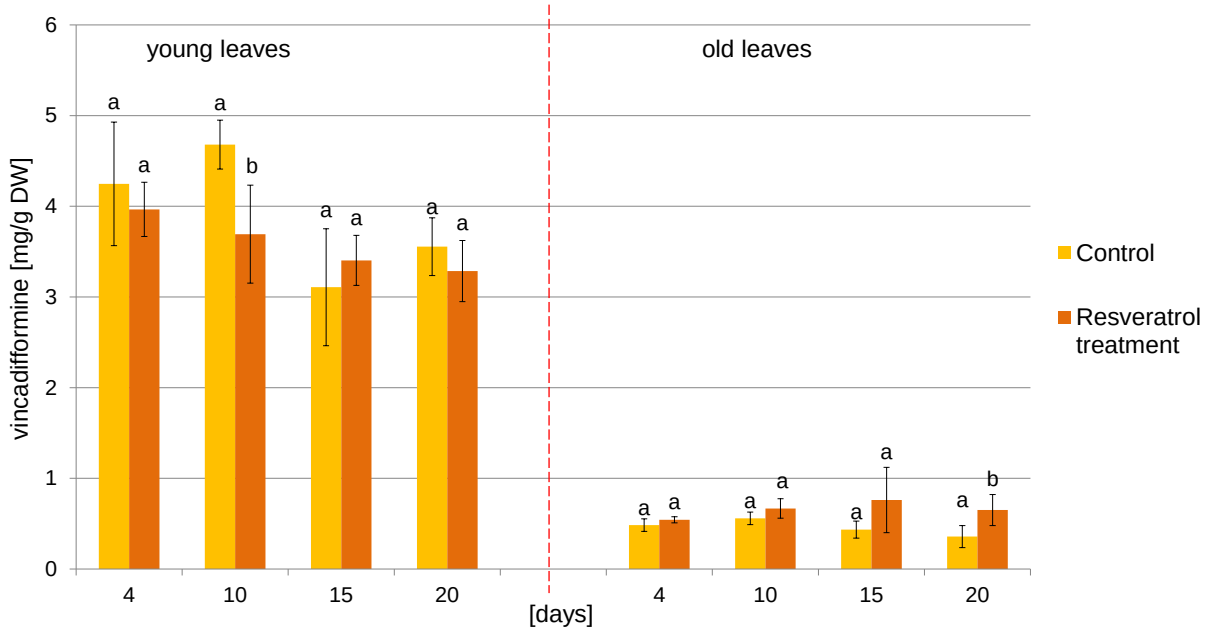


Figure 4- 58: Concentration of vincadifformine in young and old leaves. Different lower–case letters on top of the column for each period of time indicate significant differences (P≤0.05) by the least significant difference test. The mean value corresponds to the average value of six independent analyses. Error bars correspond to the standard deviation.

As already mentioned for naproxen, also the application of resveratrol primarily has no pronounced effect on the composition and concentration of the indole alkaloids present in *V. minor* plants, since the reactions sensitive for this inhibitor are absent. Consequently, also resveratrol has to be applied to plants in which the conversion of vincadifformine to minovincine is induced. Accordingly, in a further experiment, MeJA and resveratrol had been applied concomitantly.

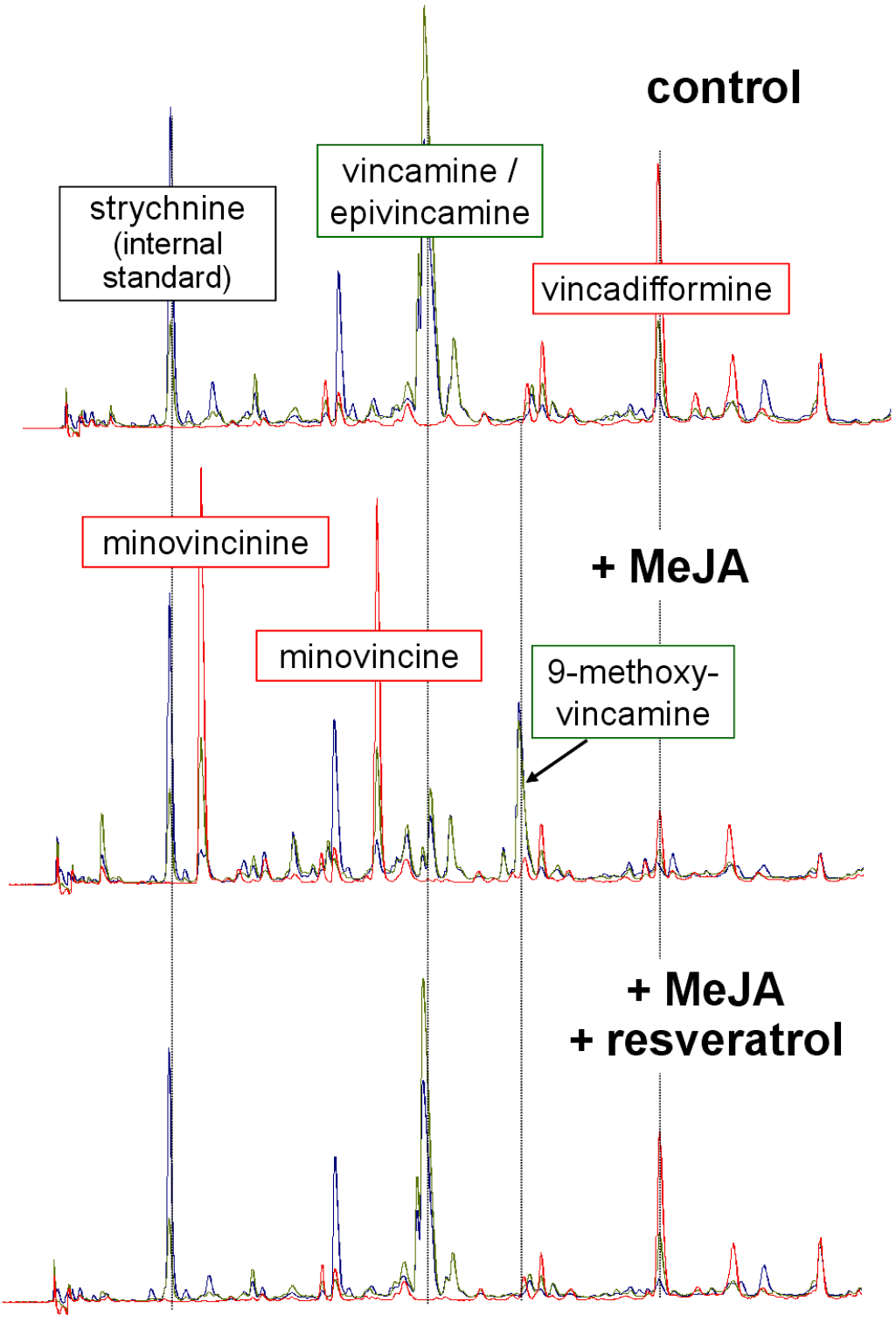


Figure 4- 59: Changes in the alkaloid spectrum of old leaves due to the application of MeJA and in combination with resveratrol to *V. minor* plants after nine days of the treatment.

In analogy to the outcome related to the naproxen application also the concomitant spaying of MeJA and resveratrol results in a very effective suppression of the MeJA induced conversion of the indole alkaloids (Figure 4-59).

The data mentioned in this chapter clearly confirm the scheme outlined in Figure 4-18 and verify the previous results, i.e., that MeJA treatment massively changes the pattern of the indole alkaloids in *V. minor* as outlined above: the concentration of vincadifformine and vincamine massively are reduced whereas the content of minovincine and minovincinine (putatively derived from vincadifformine), and that of 9-methoxyvincamine (putatively derived from vincamine), drastically are enhanced.

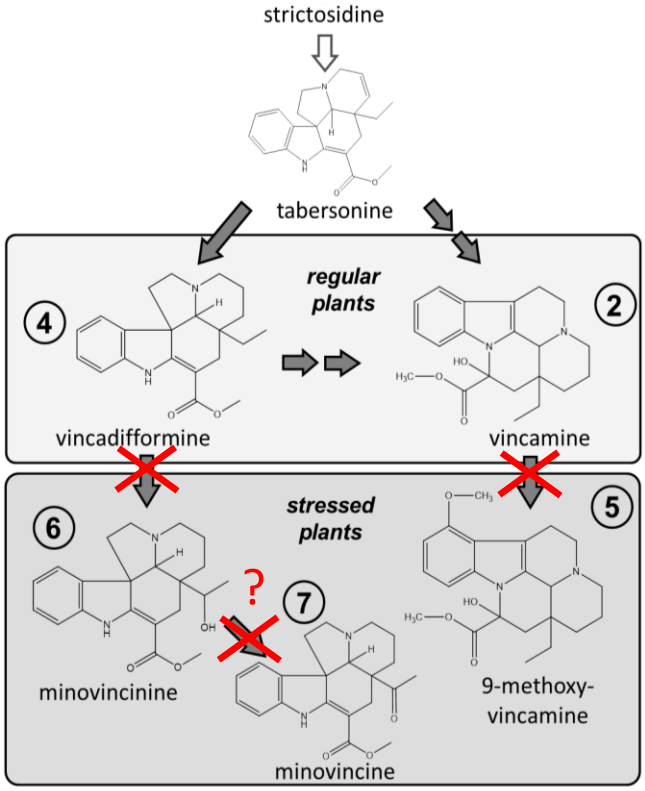


Figure 4-18a: Resveratrol inhibits the conversion of indole alkaloids in *V. minor* in response to MeJA treatment as outlined in Figure 4-18.

However, when MeJA and the inhibitors resveratrol or naproxen respectively, are applied together, the conversion of vincadifformine and vincamine does not occur (Figure 4-18a). This fascinating effect could be caused either indirectly by a suppression of MeJA related induction or directly by an inhibition of the enzymes responsible for the conversion of vincamine and vincadifformine, respectively. Indeed, due to its antioxidative properties, resveratrol is known to reduce various stress-related effects (Shin *et al.*, 2009; He and Yan, 2013; Pociecha *et al.*, 2014). Thus – in principle – this stilbene might be able to generate antagonistic effects with respect to the impact of MeJA. However, two major points favor the second option, i.e., the inhibition of the modifying enzymes by resveratrol or naproxen, respectively. First, resveratrol and naproxen are described to inhibit or substrate of various cytochrome P450 enzymes, i.e., monooxidases, hydroxylases etc. (Miners *et al.*, 1996; Chan and Delucchi, 2000; Laden and Porter, 2001; Piver *et al.*, 2001; Basheer *et al.*, 2016); secondly, the same results have been achieved, for the co-application of MeJA with both inhibitors, which reveal a quite different structure, but in contrast to resveratrol, is has no pronounced antioxidative capacity.

Summing-up - 4.4.4-4.4.5 Effect of growth regulators and inhibitors on the alkaloid concentration

1- **H_2O_2** induced a transient increase of the vincamine and vincadifformine concentrations followed by massive decrease in their content after two weeks. The time frame of the accumulation of 9-methoxyvincamine, minovincinine and minovincine coincided with the corresponding decline of its putative precursor (i.e., vincamine and vincadifformine) with underlines the assumption that vincamine is converted to 9-methoxyvincamine, and vincadifformine to minovincinine and minovincine, respectively.

2- **MeJA** induced a significant decrease in vincamine and vincadifformine concentrations, between day 4 and 8, putatively due to its conversion to 9-methoxyvincamine, minovincine and minovincinine. Subsequently, the vincamine and vincadifformine concentrations increased again presumably due to a massive biosynthesis of these alkaloids.

3- **SA** treatment resulted in a transient increase of the vincamine and vincadifformine accumulation in the leaves. Obviously, SA does not induce a conversion of vincamine to methoxyvincamine and of vincadifformine to minovincine and minovincinine, respectively.

These results show that the levels and time responses of increase of 9-methoxyvincamine, minovincinine and minovincine on the one hand and the decrease of vincamine and vincadifformine on the other hand strongly vary, depending on the growth regulators applied.

4- **Inhibitors of oxidative enzymes :**The MeJA induced changes in the alkaloid pattern of *V. minor* could be suppressed by the application of resveratrol or naproxen. Up to now, it is not known, whether the conversion of vincadifformine and vincamine is suppressed indirectly, i.e., by blocking the signal transduction or by a direct inhibition of the oxidative enzymes involved.

Chapter 5: Discussion

This thesis was aimed to comprehend the effects of methyl jasmonate (MeJA) and other growth regulators on the pattern of indole alkaloids of *Vinca minor* with a special emphasis on the appearance of the novel, so far unknown indole alkaloids. The intention was to investigate whether or not the application of growth regulators could be a novel strategy to modify the composition of active components in medicinal plants. The results presented in this work vividly demonstrate that such approach indeed leads to massive qualitative as well as quantitative variations in the content of bioactive compounds. In consequence, this outcome highlights that the utilization of growth regulators, especially of MeJA, could be a promising tool for altering and diversifying the composition of active substances in medicinal plants.

For comprehensively evaluating of this issue, two major aspects have to be considered: first, up to now, any altering and optimization of medicinal plants were focussed only on quantitative changes, and secondly, any changes in the composition, e.g., induced by various environmental impacts, were generally considered as a negative issue. The deliberate alteration of the composition of active ingredients by influencing the growth conditions illustrates a novel strategy, revealing a high potential of process innovation (Figure 5-1).

Due to the fact that most of the active compounds of well-known medicinal plants already had been extensively studied, the enthusiasm for using these plant extracts for the discovery of novel pharmaceutical leads had recently been declined massively (Raskin *et al.*, 2002; Poulev *et al.*, 2003). Moreover, today, the identification of new valuable and efficacious requires the extraction of various kilograms of plants material (Li *et al.*, 2018; Chen *et al.*, 2018; Ayoub *et al.*, 2018; Liu *et al.*, 2018; J Zhang *et al.*, 2018). In this context, the presented approach seems to be a promising alternative (Figure 5-1).

Up to now, the environmental impacts on the composition of active ingredients of medicinal plants had mostly been assessed negatively: the lack of reproducibility reported for more than 40% of related plant extracts is considered as one of the major drawbacks in using plants in pharmaceutical discovery (Cordell, 2000). Moreover, the biochemical profiles of plants harvested at different times and locations vary greatly (Poulev *et al.*, 2003; Atanasov *et al.*, 2015). Indeed, with respect to defensive compounds, such variability is not surprising, and it is well known that in addition to quantitative changes, stress may also alter the composition of the relevant compounds (Rijhwani and Shanks, 1998; Rodriguez *et al.*, 2003; Poulev *et al.*, 2003; Wojakowska *et al.*, 2013; Tang *et al.*, 2015; Sampaio *et al.*, 2016). This especially accounts for

the production of phytoalexins in response to pathogen attack, representing a typical biological stress (Ahuja *et al.*, 2012; Daniel, 2017). In consequence, the quantity, as well as the composition of healthy and non-stressed plants, could be quite different than those of the plants elicited by various biological and abiotic stress situations. However, up to now, these coherences have not been taken into account adequately, when searching for novel pharmacological active natural products. Accordingly, it seems to be obvious to exert these coherences and to develop alternative approaches to harness the stress-related alterations to deliberately vary the composition of active compounds of medicinal plants. The next level of a corresponding implementation is to mimic the related stress or developmental situation by the application of growth regulators or inductors (Figure 5-1). Consequently, in this thesis, MeJA salicylic acid, and hydrogen peroxide were used to induce characteristic stress responses to the impacts on the secondary metabolism.

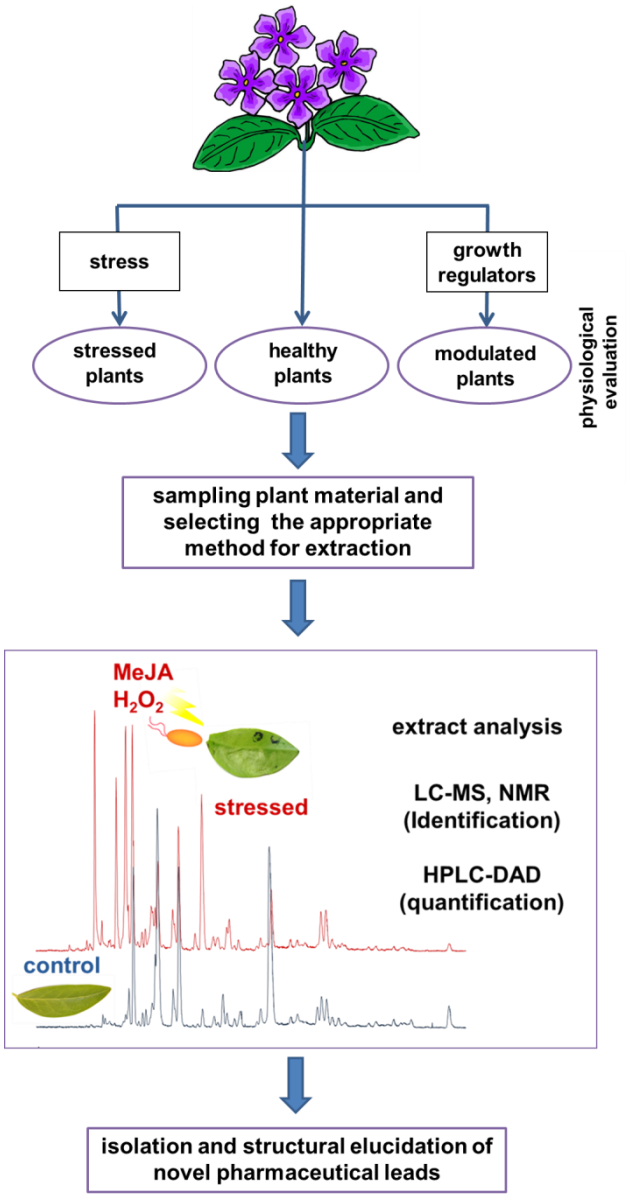


Figure 5- 1: Schematic overview of the novel approach to study the effects of stress and growth regulators as promising tools for phytochemical drug discovery.

The application of MeJA and hydrogen peroxide leads to massive changes in the pattern of indole alkaloids of *Vinca minor*, including the appearance of the novel, so far unknown indole alkaloids**.** Accordingly, MeJA treatment on *Vinca* plants was proved to be an appropriate and promising approach for identifying novel pharmacological products (Figure 5-1).

Based on the structural similarities, it is postulated that as response of the MeJA treatment vincamine is converted to methoxyvincamine and vincadifformine to minovincine and mino-vincinine (Figure 4-18). These modifications require oxidative enzymes (Rodriguez *et al.*,

2003; Giddings *et al.*, 2011). It seems to be very likely that these reactions are catalyzed by cytochrome P450 enzymes (Rodriguez *et al.*, 2003; Giddings *et al.*, 2011). This assumption is supported by the fact that the MeJA induced modifications could be suppressed by the application of resveratrol or naproxen, representing typical inhibitors or substrates for oxidative enzymes. In the following chapters, the different aspects of the various novel insights are discussed in detail.

5.1 Indole alkaloids in mature, healthy *V. minor* plants

Up to now, tremendous high number of data on the monoterpenoid indole alkaloids (MIAs) of *V. minor* and their pharmaceutical effect is available (Proksa *et al.*, 1988; Vas and Gulyás, 2005; Grujić *et al.*, 2015). The alkaloids of *Vinca minor* had been studied thoroughly by several research groups in Europe. Up to now, more than 60 indole alkaloids have been extracted, isolated from lesser periwinkle and structure elucidated (Taylor, 1965; Aynilian *et al.*, 1974; Proksa and Grossmann, 1991; Appendix Table A-1). Although different studies frequently reveal quite different compositions of indole alkaloids of *V. minor*, in all cases, the major alkaloid in the leaves was reported to be vincamine. In general, this eburnamine-type MIA accounts for 25-65% of the total alkaloids from *V. minor* (Proksa and Grossmann, 1991; D'Amelio Sr. *et al.*, 2012; Hasa *et al.*, 2013). Vincamine is a poorly soluble potent neuroprotector and cerebral vasodilator, which frequently is employed to treat CNS disorders (Vas and Gulyás, 2005; Hasa *et al.*, 2013). Hasa et al. 2013, reported that the oral bioavailability of vincamine from a standardized dry extract of *Vinca minor L.* is sevenfold higher compared to pure vincamine. As in this certain case, more than 99 % of the alkaloids are represented by the main compound vincamine, the author suggests that the better pharmacological effectivity of this standardized extract, would be due to an enhanced bioavailability. Hasa et al. (2013) postulated that the reason for this effect might be due to an interaction of the alkaloids with other compounds, e.g., flavonoids, which - in addition to vincamine – are also present in the corresponding standardized extracts. However, when inspecting the corresponding TIC of the standardized extract, no hints for the presence of these compounds can be found. Nonetheless, also in other cases, the differences in bioavailability have been attributed to specific interactions resulting in a so-called 'phytocomplex', e.g., for khellin and the corresponding crude extracts from *Ammi visnaga* (Eder and Mehnert, 2000). The authors deduced that the enhanced bioavailability of the co-administrated compound is either due to an enhanced solubility of the 'phytocomplex' or an increased rate of its absorption. Accordingly, the use of dried standardized extracts could be an attractive alternative to the administration of purified vincamine for

treatment of memory disorders or as a CNS stimulant. However, such approach requires exact conditions for the standardization procedure.

It is well known that the amount of Lesser Periwinkle's alkaloids, and particularly that of vincamine, varies to a large extent as a function of the climate, the general growth conditions and the season of herb harvesting (Boyadzhiev *et al.*, 2002). In general, the content of alkaloids reaches its maximum at the flowering stage (Farahanikia *et al.*, 2011). In addition to these investigations employing the genuine plants, much further research had been performed using a related tissue and organ cultures (Verma *et al.*, 2012; Verma *et al.*, 2014a; Verma *et al.*, 2014b). Indeed, these approaches have no direct relevance for pharmaceutical aspects, but they could help to understand and elucidate biosynthetic pathways and their regulation. Notwithstanding, for the discovery of novel plant drugs, the investigation of genuine plants are inevitable, since the synthesis of bioactive secondary metabolites frequently depends on the interactions with the environment, e.g., abiotic and biotic stress factors. Moreover, the production of natural products is restricted to certain tissues or organs due to tissue-specific gene expression patterns of the enzymes responsible for the secondary metabolism. These coherences may explain, why most cell or tissue cultures, representing dedifferentiated cells, commonly reveal only very low levels of secondary metabolites and thus exhibit only a little or no pharmacological activity (Littleton, 2007). In addition, the vincamine content in the mature leaves of *V. minor* was about 0.13% (Proksa and Grossmann, 1991), whereas the corresponding tissue culture contained only about one third (0.041%; (Hou *et al.*, 2011)). Nevertheless, in very few cases cultured plant cells produce secondary metabolites and are used as potential sources for phytopharmaceuticals. Further problems concerning the production of metabolites by cell cultures are related to their low growth rate, various problems in scaling-up and the instability of selected cell lines (Whitmer *et al.*, 2003). Considering all these coherences, this thesis was based on an investigation of genuine, mature *Vinca minor* plants.

As known for other alkaloids, the content of MIAs content in *V. minor* varies seasonally (Kaczmarek and Lutomski, 1965) Plants collected between the middle of April and June contained the most content of alkaloids with hypotensive activity, and therefore are most suitable for pharmaceutical purposes. Moreover, Boyadzhiev et al. (2002) reported that the vincamine content strongly depends on the growth and climatic conditions, and putative stress situations and the highest content of alkaloid was detected at the beginning of flowering and at fruiting time (Girre, 1971). Furthermore, Mathe (1965) reported that the total alkaloid and vincamine contents of the leaves were higher when the *V. minor* plants were grown under

sunny, shadeless conditions, rather than in shaded areas. Thus, although these plants prefer to grow in shaded areas, they should be grown in open fields to increase the vincamine yield.

Accordingly, in this study, which is aimed to elaborate a new approach for phytochemical drug discovery, only mature *V. minor* plants of defined physiological status were used. Furthermore, to investigate the effect of MeJA treatment and of application of another growth regulator on the composition of alkaloids, only plants cultivated between April and June were employed. When analyzing these intact and healthy plants, always four main alkaloids, i.e., vincamine, vincadifformine, and strictamine and 10-hydroxycathafoline were detected. These alkaloids represent the three major groups of MIAs (Figure 5-2).

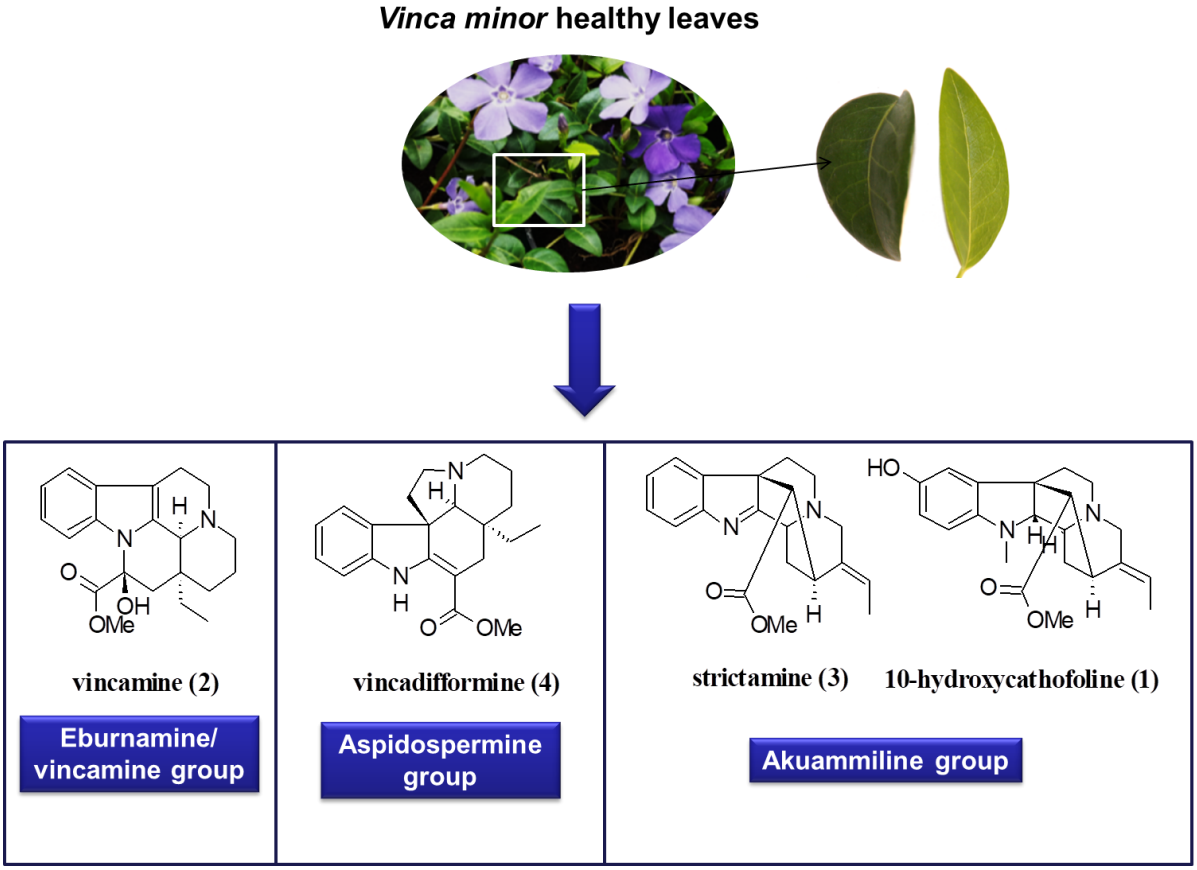


Figure 5- 2: The major indole alkaloids determined in *V. minor* control plants leaves.

Whereas vincamine, vincadifformine, and strictamine already had been reported several times to be present in *V. minor* (Proksa and Grossmann, 1991; D'Amelio Sr. *et al.*, 2012), the forth alkaloid was described to occur for the first time in this plant. Based on a comprehensive structure elucidation (corresponding NMR data are mentioned in the appendix, Figure A-2: A-9) this alkaloid was identified as 10-hydroxycathofoline. In this context, it is worth mentioning that this alkaloid is absent in young leaves and occurs only in traces in old leaves, which are harvested in late summer. On the first sight, these facts seem to approve the occurrence of

seasonal variation as reported by Balsevich et al. (1982). However, it has to be taken into consideration, that such variation, e.g., the absence of 10-hydroxycathofoline, also could be due to differences in the developmental and or maturation stage of the plants analyzed.

The same coherences pertain to the other main alkaloids. Whereas – as outlined in result chapter (4.2) in detail - vincamine represents the major alkaloid in old leaves of *V. minor*. In contrast, in young leaves, vincadifformine is the alkaloid exhibiting the highest concentration. Accordingly, the total content of vincamine strongly increases whilst leaf maturation, whereas that of vincadifformine remains mostly constant. Obviously, the accumulation of these both alkaloids is controlled differentially.

In general, metabolism and metabolic actions are impacted multifariously, including developmental, ontogenetic, spatiotemporal, and environmental factors (Kursar *et al.*, 2009; Barton and Koricheva, 2010; Kooke and Keurentjes, 2012; Richards *et al.*, 2015; Wiggins *et al.*, 2016) Figure 5-3.

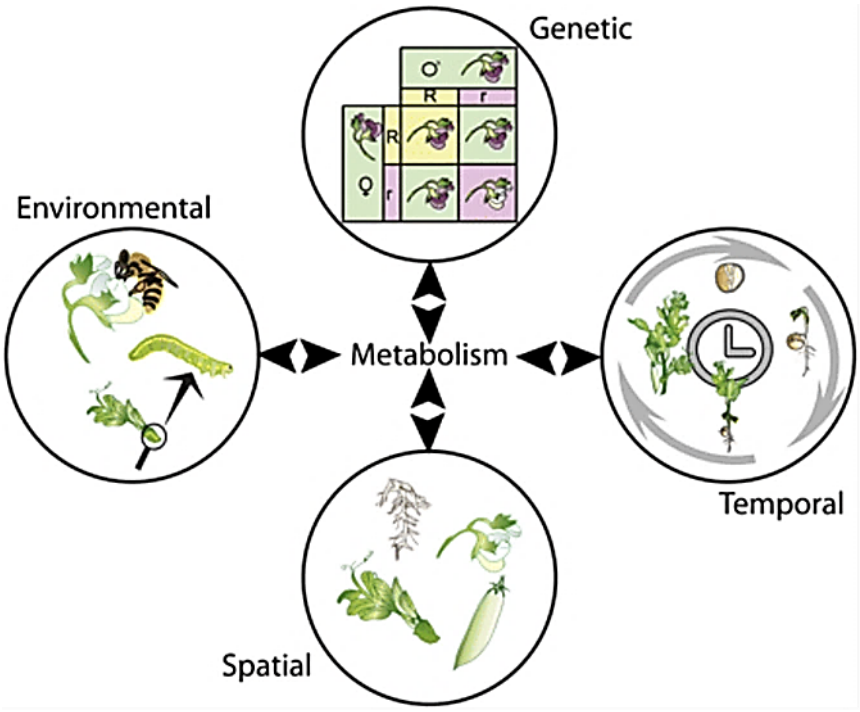


Figure 5- 3: The multifactorial regulation of plant metabolism. The interplay between temporal, spatial, environmental, and genetic factors determines the quantitative and qualitative profile of a plant's metabolome (Kooke and Keurentjes, 2012).

Koricheva and Barton, (2012) proposed a model to explain the variation and temporal changes especially in secondary metabolite patterns and its concentration by introducing the terms proximate and ultimate causes. According to Koricheva and Barton, (2012), proximate causes comprises all mechanistic-based processes, such as developmental and environmental influences that impact on the generation of chemical diversity, whereas the ultimate causes are

related to evolutionary, phylogenetic issues. As this thesis is focussed on putative approaches to impact the concentration of secondary metabolites, i.e., alkaloids, only the issue of proximate causes will be discussed in detail.

a) Proximate causes of temporal changes in SM concentrations

The increase or decline of the content of a certain secondary metabolite in plant tissues always results from the difference in its gain and its loss. This balance is always determined by the rates of biosynthesis of a certain substance, its allocation, and its modification or degradation, respectively (Figure 5-4), which, in turn, might be impacted to a different extent by developmental, ontogenetic, spatiotemporal, and environmental factors (Figure 5-3).

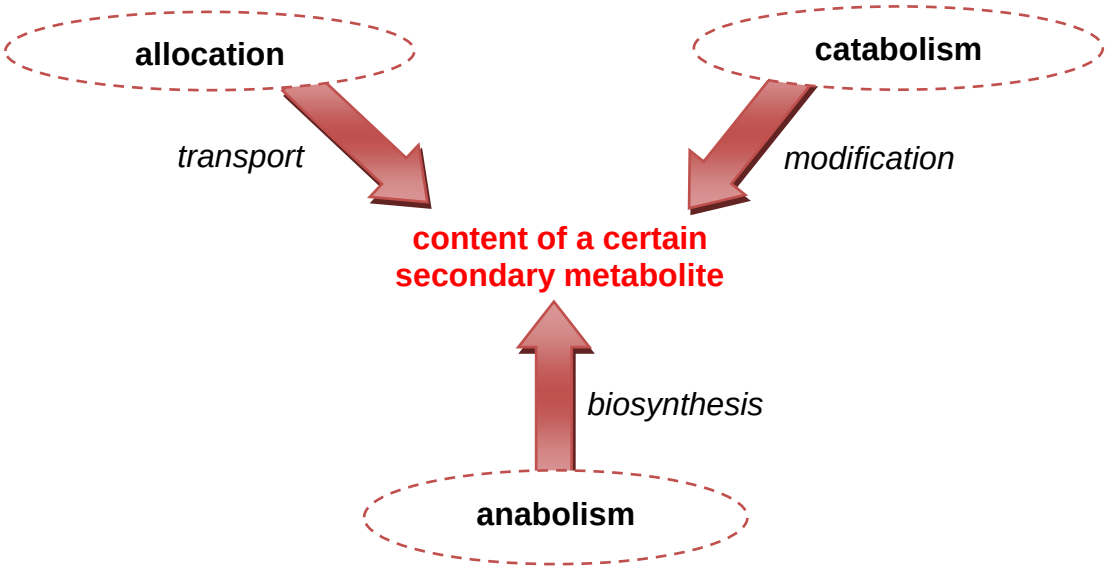


Figure 5- 4: Proximate causes of temporal changes in content and composition of secondary metabolites

Impact on biosynthesis

In *Mentha* × *piperita* (Lamiaceae), a plant used as model system for the study of monoterpene metabolism, the rate of biosynthesis was shown to be the principal factor for increasing monoterpene contents in young leaves, whereas rates of monoterpene losses through volatilization or catabolism were negligible (Gershenzon *et al.*, 2000). Moreover, this study showed that monoterpene biosynthesis in *M. piperita* is restricted to an early period in leaf development. This is in accordance with the finding that the activities of the enzymes responsible for monoterpenes biosynthesis and the expression of the corresponding genes in *M. piperita* had very similar developmental profiles (McConkey *et al.*, 2000).

If the biosynthesis of a certain secondary metabolite occurs predominantly in early in leaf development and stops before leaf expansion is completed, the total content of this natural

product remains constant, whereas its concentration (based on leaf area, dry or fresh weight) will strongly decrease in the course of leaf maturation (Koricheva, 1999). In this sense, in leaves of a tropical tree *Inga umbellifera* (Fabaceae) flavonoids are synthesized only until an intermediate expansion stage whereas the leaf biomass continues to increase until the leaf is fully mature; as result, the flavonoid concentrations decline although the total content of flavonoids per leaf remains the same (Brenes-Arguedas *et al.*, 2006). In contrast, in another Inga species, i.e., in *I. goldmanii*, such significant dilution in the flavonoid concentration is not detectable. In this plant, the synthesis of flavonoids proceeds throughout the entire leaf development, indicated by a continuous increase in the total content of flavonoids. In consequence, although growth related increase of biomass and leaf area occurs, flavonoid concentrations is not diluted or results in a much lesser extent than in *I. umbellifera* (Brenes-Arguedas *et al.*, 2006; Wiggins *et al.*, 2016).

The results related to the contents of indole alkaloids *in V. minor* presented in this investigation, reveal that both of the options mentioned above could be realized in on plant: the concentration of vincadifformine is markedly lower in old leaves than in young ones, putatively due to a corresponding dilution of the alkaloid by the massive gain of biomass per leaves. In contrast, the concentration of vincamine is significantly enhanced in old leaves, verifying a considerable rate of vincamine biosynthesis whilst leaf maturation.

Impact on allocation

In addition, to alteration in the rate of biosynthesis, temporal changes in secondary metabolite contents may also be due to allocation processes, i.e., their transport from one plant tissue or organ to another. A famous example for such coherence is the decrease in the concentrations of pyrrolizidine alkaloids in older leaves of *Cynoglossum officinale* (Boraginaceae). As any catabolism of this compounds was ruled out, the decline of the alkaloid content could only be due to its translocation from the mature leaves - acting as a physiological source - into young developing leaves (Vandam *et al.*, 1995). In analogy, in *Senecio* species (Asteraceae), pyrrolizidine alkaloids are synthesized in roots and are translocated - in the form of the corresponding alkaloid *N*-oxide - into the *sink* organs like flowers, seeds, and fruits (Hartmann and Dierich, 1998). This translocation of alkaloids into the *sink* organs always is realized via the phloem. However, in some cases, translocation of alkaloids is also performed via the xylem. However, all substances transported within the xylem will not be translocated into *sink* organs, but instead only into those tissues exhibiting significant rates of transpiration, i.e., predominantly into the leaves (Nowak and Selmar, 2016). The best example for such allocation

is the transport of nicotine. This pyrimidine alkaloid is synthesized in tobacco roots and subsequently translocated via the xylem. This allocation results in its accumulation in the transpiring leaves, whereas only traces of nicotine are translocated from the root into the typical physiological *sink* organs, e.g. the flowers, seeds or fruits (Nowak and Selmar, 2016). In consequence, the extent of accumulation of nicotine in the leaves predominantly is affected by the respiration rate of the leaves, which in turn strongly depends on the temperature and humidity and thus on varying seasonal situations.

Indole alkaloids of *V. minor* are also present in the in the roots (Proksa and Grossmann, 1991), fruits, and flowers (Appendix Figure A-70). However, each organ exhibits a distinct spectrum of terpenoid indole alkaloids, (MIAs), especially when comparing leaves and roots. Similarly, in *C. roseus* also different patterns of indole alkaloids were reported for various tissues (e.g., the bisindole alkaloids are restricted to the aerial parts of the plant and do not occur in the roots). In *C. roseus*, the distribution of the different types of alkaloids nicely correlates with the occurrence of the specific enzymes responsible for their biosynthesis, and the expression of the corresponding genes, respectively (Laflamme *et al.*, 2001). For example, vindoline is accumulated exclusively in the laticifer and idioblast. In addition to the enzymes responsible for catalysis of the late steps of vindoline biosynthesis indeed are associated exactly in these cells. In contrast, the enzymes catalyzing the biosynthesis of minovincinine and hörhammericine, which are characteristic for the root tissue, indeed only are present in this organ (Laflamme *et al.*, 2001). Accordingly, based on this congruences, it is deduced for *C. roseus* that the biosynthesis of a certain alkaloid takes place in that tissue, in which it is accumulated; in consequence, major contributions of allocation are thought to be very unlikely (St-Pierre, 1999; Shukla *et al.*, 2006).

However, on cellular level, various transport steps of MIAs are realized, since – at least in *C. roseus* - MIA biosynthetic is compartmented at cellular as well as subcellular levels: the enzymes involved in the MIAs biosynthesis are located in different cell types and their interaction requires intercellular translocation of intermediates (Burlat *et al.*, 2004; Guirimand *et al.*, 2011).

In conclusion, the transport of indole alkaloids in *C. roseus* is assumed to occur at an intercellular level rather than between different organs. However, no sound statements can be deduced with respect to a putative translocation of MIAs from leaves or roots into reproductive organs. Up to now, less information on the occurrence of indole alkaloid biosynthesis in the fruits and seeds of *C. roseus* was reported (Eilert *et al.*, 1985). Yet, if we deduce that in analogy

to other alkaloidal plants the alkaloids accumulated in these *sink* organs are – at least in part - synthesized in *source* organs and are translocated via phloem into the fruits and seeds. Vivid examples for such courses of events are the pyrrolizidine alkaloids in *Senecio* (Hartmann and Dierich, 1998), also the quinolizidine alkaloids in *Lupins* (Wink and Witte, 1984; Lee *et al.*, 2007; Frick *et al.*, 2017) and tropane alkaloids in solanaceous plants (e.g. *Hyoscyamus niger*; (Hashimoto *et al.*, 1991).

In the case of a putative translocation of MIAs in the phloem of *V. minor*, these alkaloids are exposed to the same constraints as the other types of alkaloid: the phloem is reported to be slightly basic (pH 8; (Tammes and van Die, 1964; Nowak and Selmar, 2016)). Accordingly, most of the alkaloids will be present as unprotonated free bases, which – in general – are able to pass see biomembranes. In contrast, the xylem is acidic, a milieu in which most alkaloids are protonated and in result are not anymore able to pass biomembranes. In consequence, the alkaloids would be trapped in the xylem according to the ion-trap mechanism outlined first by Matile, (1976). Only the constantly charged quaternary alkaloids will not be affected by this process. To prevent a bleeding of secondary and tertiary alkaloids, they are converted to corresponding *N*-oxides (Nowak and Selmar, 2016), which are not able to diffuse through membranes. Only in the case of a phloem transport of very alkaline alkaloids the generation of such transport metabolites is not required since these alkaloids are protonated even in the slightly alkaline phloem. In this manner, the occurrence of quinolizidine alkaloid in the phloem of *Lupins* is coherently explained. In contrast, MIAs reveal much lower alkaline properties and consequently, in order to be translocated from *source* to *sink* organs via phloem, special transport metabolites like *N*-oxides should be required. In this sense, further investigations on the occurrence of indole alkaloid N-oxides would be helpful in order to elucidate whether or not MIAs are translocated within *V. minor* plants. Nevertheless, the presence of some corresponding *N*-oxides and some quaternary indole alkaloids in *V. minor* (Votický *et al.*, 1979; Proksa *et al.*, 1989) and other members of Apocynaceae (Cao *et al.*, 2012; Zhang *et al.*, 2013) are the first hint that also – at least some – MIAs also are translocated from *source* to *sink* organs. As consequence, also putative changes in the extent of such translocation which in turn could also be affected by developmental stages and environmental conditions, have to be taken into consideration when evaluating and assessing corresponding changes in the alkaloid content in various organs.

Impact on modification or degradation

The third factor which might be responsible for changes in the content of SMs is related to the conversion of the natural product. The extent of such modifications might range from slight and minor modifications (e.g., Steinegger and Bernasconi, 1964; Waller and Skursky, 1972) up to a complete degradation (e.g., Kalberer, 1965).

An entire breakdown of natural products is known for various classes of natural compounds: cyanogenic glucosides are converted to primary metabolites during the seedling development of *Hevea brasiliensis* (Selmar *et al.*, 1991), cyanogenic lipids are degraded whilst seedling development of *Ungnadia speciosa* (Selmar *et al.*, 1990), and canavanine is metabolized in seedlings of *Canavalia ensiformeis* (Rosenthal, 1970). In addition, many papers are available reporting also a degradation of alkaloids, e.g., of a major share of pyrrolizidine alkaloids is metabolized during germination of *Crotalaria scassellatii* (Wink and Witte, 1985; Toppel *et al.*, 1988), and caffeine and related purine alkaloids are degraded in the leaves of *Coffea arabica* (Ashihara *et al.*, 1996). In some cases, the observed degradation correlates with certain changes in developmental stages, e.g., seed germination, whereas for other plants an ongoing degradation has been reported (Kalberer, 1965; Mothes *et al.*, 1965; Waller and Nowacki, 1978).

If a permanent degradation co-occurs with a significant rate of biosynthesis, a classical *turn over* situation results. However, if either biosynthesis or degradation prevail, this outbalance results in an increase or a decline of the alkaloid content. In this sense, as outlined above, the alkaloids stored in the seeds are mobilized during germination (Waller and Nowacki, 1978; Wink and Witte, 1985). In contrast, the seeds of *C. roseus* virtually no alkaloids are present, and during germination, alkaloids are synthesized and reach a maximum at the third week (Mothes *et al.*, 1965). Subsequently, they almost disappear within the fourth and eighth week of seedling development, before they finally reappear in older plants (Mothes *et al.*, 1965). Such observations seem to indicate a dynamic role for the alkaloids in *C. roseus*, a statement, which was verified by the results of Daddona et al. (1976). In the same sense, also in tobacco plants such extensive breakdown of nicotine and the re-entry into primary plant metabolites have been observed (Leete and Bell, 1959; Robinson, 1974).

Whereas reports on a complete degradation or a steady turn-over of alkaloids are relatively rare, the modification of alkaloids seems to be far more common. Nevertheless, albeit in the course of their biosynthesis nearly alkaloidal basic structures are modified all, i.e., each of the synthesized compounds in the metabolic pathway serves as a precursor for the subsequent one,

these phenomena are not considered as "modification of alkaloids". However, if a certain alkaloid is accumulated temporarily and later on, e.g., at a specific developmental stage, is modified, it is implemented in this classification. In this sense, the growth-related dimerization of vindoline and catharanthine into anhydrovinblastine in leaves of *C. roseus* (Naaranlahti *et al.*, 1991) has to be evaluated as a modification of accumulated alkaloids. In the same manner, the alkaloid transformations in *Genista aetnensis* during a growing season reported by Steinegger and Bernasconi, (1964) have to be rated as SM-modifications: during plant development, the level of sparteine decreases while that of retamine increases, caused by the oxidation. In parallel, methylcytisine is demethylated to cytisine and further on transformated to anagyrine (Steinegger and Bernasconi, 1964). In analogy, also methylation demethylation may also play an important role in mobilization and translocation of ricinine (Skursky *et al.*, 1969): in mature castor bean leaves, ricinine accumulated. In the course of senescence, it is converted to N-demethyl ricinine, which is translocated in the xylem via the transpiration stream (Waller and Skursky, 1972). In this sense, also the MeJA induced modifications of indole alkaloids presented in this thesis, are vivid examples of typical modifications of alkaloids.

Apart from ordinary and plain conversions, alkaloids might be modified by the formation of complexes with molecules of different classes of plant secondary metabolites. In this context, the stacking interaction of caffeine and chlorogenic acid in coffee or caffeine and catechin in tea represents the most popular examples (Colon and Nerin, 2014; D'Amelio *et al.*, 2015). In addition to such simple stacking interaction, alkaloids may also modify by covalent bonds, e.g., by the attachment of a glucose moiety, as it is reported for glycoalkaloids in *Solanum* species (van Gelder *et al.*, 1988) and indole alkaloid glycosides isolated from *Uncaria tomentosa* (Kitajima et al. 2000) *and C. roseus* roots (Kitajima *et al.*, 2000; Wang *et al.*, 2012)**.**

In conclusion it could be stated that not just the total content of alkaloids, but also their composition may change through plant development; a phenomenon which also is reported for many other secondary metabolites, such as monoterpene, phenolics and flavonoids (Gershenzon *et al.*, 2000; Salminen *et al.*, 2001; Salminen *et al.*, 2004; Sampaio *et al.*, 2016; Wiggins *et al.*, 2016).

Impact of environmental and developmental cues and challenges

In addition to the ontogenetic changes mentioned above, also environmental factors, e.g., changes in temperature, the availability of light (photoperiod), water and nutrients and various stress situations affect the composition and extent of the secondary metabolites. These, in fact,

are determined by rates of biosynthesis of natural products, its allocation, and its modification or degradation, respectively Figure 5-4. As each of this process might be influenced in a certain manner either by environmental or by developmental factors, the entire situation with respect to the elucidation of putative impacts of the various factors and the related regulation mechanisms is very complex. Indeed, in principle, it is possible to verify an active turn over or putative translocation of a certain substance by using radioactively labeled compounds. In the same manner, by applying labeled biogenetic precursors the extent of biosynthesis could be evaluated, conceivably in combination with related studies on the expression of biosynthetic enzymes. However, it is very unlikely to comprehensively consider all these processes adequately and to identify the various different effects of all environmental and developmental factors. Accordingly, most studies just concentrate on the overall changes and do not distinguish between the certain impacts on biosynthesis, allocation and metabolic degradation. Moreover, in general, only a few of the numerous environmental and developmental factors, which are impacting the secondary metabolism, could be investigated in parallel. These coherences always have to be considered when certain changes and modification should be evaluated.

In principle, these difficulties are inherent and fundamental in nearly all corresponding investigations, which might be outlined by a few examples: In annual and short-lived herbaceous plants, seasonal and ontogenetic changes are confounded, such as leaf expansion usually occur within a relatively short time interval during the season (e.g. a few weeks in spring in temperate ecosystems). Nevertheless, the seasonal change (different temperature, the ratio of light/dark) strongly influences plant development, especially induction of flowering and in consequence production of seeds and change in *source-sink* properties. For instance, the rate of purine alkaloids biosynthesis in tea leaves (Fujimori *et al.*, 1991), and indole alkaloid brachycerine biosynthesis in *Psychotria brachyceras* was varied between different seasons and seedling development (Gregianini *et al.*, 2004).

Moreover, plants interact with various and complex biotic and abiotic factors, like temperature, light, water availability, pest, and herbivores. Yet, these numerous environmental factors frequently are influencing and impacting each other. In this sense, for example, a high irradiation is frequently accompanied with elevated temperatures, high irradiation often parallels with UV-radiation, and elevated temperatures co-occur with higher evaporation rates (Kleinwächter and Selmar, 2014). Moreover, some parameters, such as drought, impact also on the entire ecosystem and might be associated with a higher herbivore pressure, but a lesser number of pathogens.

In conclusion, neither an unequivocal differentiation between putative effects on biosynthesis, allocation or metabolic degradation of a certain compound by developmental or by environmental factors could be made.

b) Ultimate causes of temporal changes in SM production

Although - as outlined above – the proximate causes of temporal changes of SMs are very complex and difficult to cover comprehensively – their basis are relatively well understood (Koricheva and Barton, 2012). In contrast, the comprehensions of the ultimate causes, which in principle cover the evolutionary aspects, are still heavily debated. Two types of hypotheses have been put forward to explain the evolution of patterns of seasonal and ontogenetic variation in SM production. The first hypothesis considers the above patterns to be by-products of resource allocation constraints. Since, young leaves are more susceptible to herbivores and pathogens than mature leaves (Coley, 1983, 1983; Coley and Kursor, 1996), it is postulated that plants would benefit by allocating more resources to defend those organs (Feeny, 1992). The second hypothesis explains the evolution of temporal changes in SMs mainly with regard to adaptive responses to the selection pressure imposed by herbivores.

In this context, however, it is neglected that the presence of a certain substance might reveal various consequences. Meanwhile, there are many examples of such multifunctionality of natural products. For instance, flavonoids (e.g. lutein and zeaxanthin) absorb harmful UV-light, and also protect the chloroplast from the high light intensities and thus against light-induced damage, but in addition, they may act as radical scavengers, too (Demmig-Adams and Adams, 2002). *C. roseus* habituated to the fungal-rich tropical rainforest. *C. roseus* accumulates a complex variety if indole alkaloids, which seems to be protective not only against fungal pathogens but also are active in repelling potential herbivores (van der Heijden *et al.*, 2004).

Conclusions and implication

Based on the insights mentioned above, it can be concluded:
Any changes in the content and the composition of natural compounds always results from the balance of biosynthesis, allocation and degradation. These three processes are influenced differentially by developmental and environmental factors. Thus, their overall impact on the spectrum and content of the secondary metabolites might be very complex and multifarious.

These considerations open novel doors for manifold and deliberate approaches to change and adapt the secondary metabolism, intended to improve and optimize the search for novel pharmacological active compounds.

With respect to environmental factors, the generation of various stress situations and the concomitant consideration of the developmental stage of the plant seems to be the most promising approach. In this sense, the augmentation of capabilities combined with higher reproducibility should be achieved by the employment of growth regulators or signal transducers involved in the control of stress responses.

5.2 The effect of growth regulators on the alkaloid biosynthesis in *V. minor* leaves

Poulev et al. (2003) reported that the application of various growth regulators strongly enhances the pharmacological efficiency of corresponding plant extracts and might be a promising new approach in drug discovery. Meanwhile, it is well established that elicitation induces major qualitative and quantitative biochemical changes in the chemical composition of the plant's natural products (Poulev *et al.*, 2003; Ncube *et al.*, 2012; Wojakowska *et al.*, 2013; Wang *et al.*, 2015; Shakya *et al.*, 2017). These changes seem to be specific for each growth regulator, resulting in quite different chromatographic fingerprints of the extracts obtained from untreated or induced plants. Moreover, the corresponding changes may strongly vary with respect to the plant species investigated (Poulev *et al.*, 2003).

Up to now, many investigations on the impact of elicitation by different growth regulators on the monoterpenoid indole alkaloids (MIAs) biosynthesis have been performed (Aerts *et al.*, 1994; Zhao *et al.*, 2001a; Rodriguez *et al.*, 2003; Sottomayor *et al.*, 2004; Gregianini *et al.*, 2004; Tang *et al.*, 2009; Wei, 2010; Giddings *et al.*, 2011; Wang *et al.*, 2011). These studies clearly illustrated that methyl jasmonate (MeJA), hydrogen peroxide (H_2O_2) and salicylic acid (SA) have a great impact on MIAs biosynthesis. Unfortunately, there are many contradictions

with respect to the effect of the underlying elicitation processes (Aerts *et al.*, 1994; Aerts *et al.*, 1996; Godoy-Hernández and Loyola-Vargas, 1997; El-Sayed and Verpoorte, 2005; Pan *et al.*, 2010). Nonetheless, the authors considered these approaches as promising strategies for MIAs production and proposed forthcoming investigations, to elucidate the putative effects of MeJA, H_2O_2, and SA on modulating the composition of MIAs biosynthesis in *V. minor*. Based on the coherences unveiled in this study, many of these inconsistencies could be - at least in part - elucidated and will be discussed comprehensively in the next section. Owing to the considerations mentioned above, it is vital to consider the physiological status of the plants investigated. By doing so, the effects of MeJA or other signal molecules could be segregated from the effects of endogenous jasmonates produced due to natural stress situations or due to aging and senescence processes.

Implementing these considerations, in this thesis - as outlined in details in results section - it could be shown for the first time that the application of growth regulators and signal transducers (e.g. MeJA and H_2O_2) not only altered the overall content but also resulted in massive modification of the alkaloid pattern. Based on reverse changes in their concentration, it was deduced that vincadifformine is transformed first to minovincinine and finally to minovincine. In the same manner, vincamine is converted via 9-hydroxyvincamine to 9-methoxyvincamine (Figure 4-18, Figure 5-5). The discovery of 9-methoxyvincamine as a novel natural product further outlines that even well-known, extensively studied plants such as *V. minor*, might be an auspicious source for the discovery of novel phytochemical drugs when certain stress situation are deliberately induced.

Methyl jasmonate (MeJA) is a part of numerous signal transduction chains, including the elicitation of phytoalexins (Tamogami *et al.*, 1997; Konan *et al.*, 2014), the enhancement of protective secondary metabolites in response to various environmental factors such as insect attacks (Okada *et al.*, 2015), or abiotic stress situations (Ahmad *et al.*, 2016). In addition, MeJA is involved in the induction of senescence (He *et al.*, 2002; Qi *et al.*, 2015). Accordingly, the application of MeJA will induce many different metabolic events, which cannot be attributed to only one unique stress situation. Moreover, this elicitation, i.e., the impact of the MeJA application, is strongly affected by the physiological status of the plants (Wasternack *et al.*, 2013; Wasternack, 2014; De-Ollas and Dodd, 2016). Notwithstanding, elicitation generally causes qualitative as well as quantitative changes in the content of bioactive secondary metabolites (Poulev *et al.*, 2003; DeGeyter *et al.*, 2012; Wojakowska *et al.*, 2013; Tang *et al.*, 2015; Sampaio *et al.*, 2016).

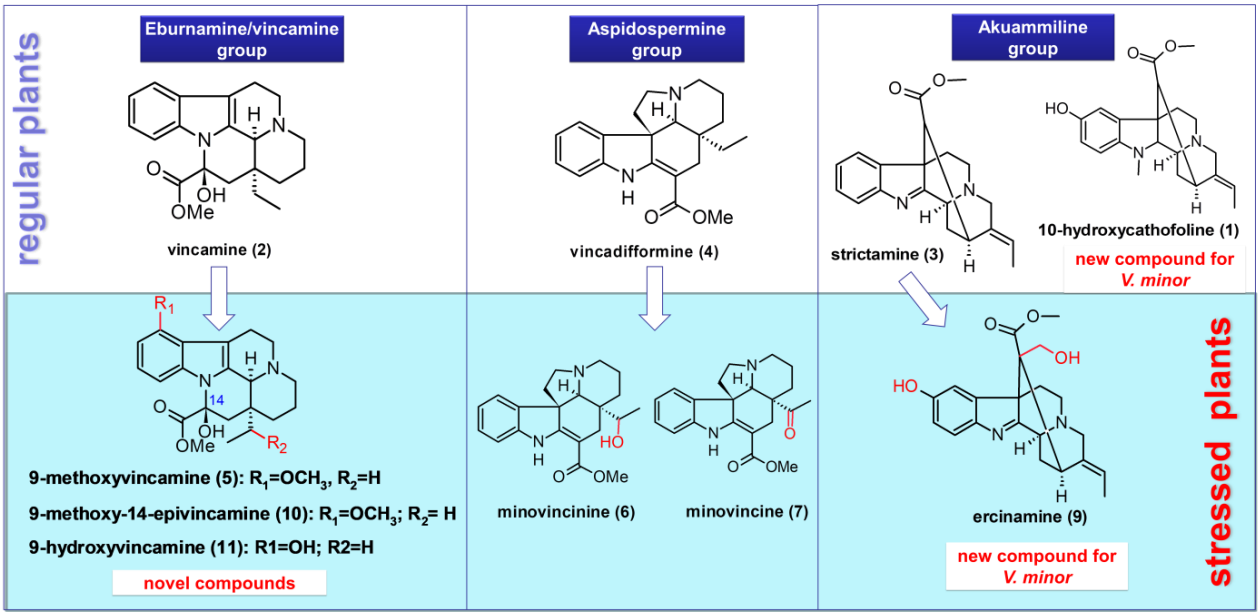


Figure 5- 5: Modification of indole alkaloids in *V. minor* after MeJA-treatment (in red: compounds, which for the first time had been demonstrated to be present in *V. minor*).

As outlined, these alterations could be due to changes in all three metabolic processes outlined above, i.e., the biosynthesis, allocation, and degradation of MIAs and, generally are related and due to changes in the expression of the corresponding enzymes (Rodriguez *et al.*, 2003; Wei, 2010; Giddings *et al.*, 2011; Wang *et al.*, 2011). Unfortunately, in the literature frequently, only the biosynthetic aspect is considered (Aerts *et al.*, 1994; Gershenzon *et al.*, 2000; Pan *et al.*, 2010; Xing *et al.*, 2011; Xiang *et al.*, 2015).

The results presented in this thesis clearly show that the modifications are the main cause of the temporal changes in the content and composition of indole alkaloids in *V. minor* leaves Figure 5-5. In principle, there might be various reasons for these modifications, e.g., they might reveal a defensive role against various environmental stress situations such as drought, herbivore or pathogen attack (Poulev *et al.*, 2003; Sampaio *et al.*, 2016). Moreover, they might be required for translocation, e.g., for the transport from *source* to *sink* organs (Nowak and Selmar, 2016), or in the course of senescence from dying into young leaves (Waller and Skursky, 1972).

Indeed, an unequivocal confirmation for the occurrence of such conversions should be provided by classical pulse-chase experiments using ^{13}C or ^{14}C labeled alkaloids, which could be generated by feeding the corresponding labeled precursors (tryptophan) to *V. minor* plants before MeJA treatment. However, the inverse changes in their concentration immediately suggest that vincadifformine is converted first to minovincinine and finally to minovincine. In the same manner, vincamine is thought to be converted to 9-methoxyvincamine (Figure 4-18b).

With respect to any modifications, especially oxidative ones, the corresponding reactions very often are catalyzed by cytochrome P450 enzymes (Rodriguez *et al.*, 2003; Wei, 2010; Giddings *et al.*, 2011; Wang *et al.*, 2011). This assumption is supported by the inhibition of these modifications when the plants concomitantly are treated with MeJA and resveratrol or naproxen, respectively. As outlined in the next chapter, resveratrol as anti-oxidant competitive substrate and naproxen as oxygenase inhibitor hamper the activity of the enzymes induced by the MeJA treatment. Accordingly, the corresponding changes outlined in Figure 4-18b are suppressed.

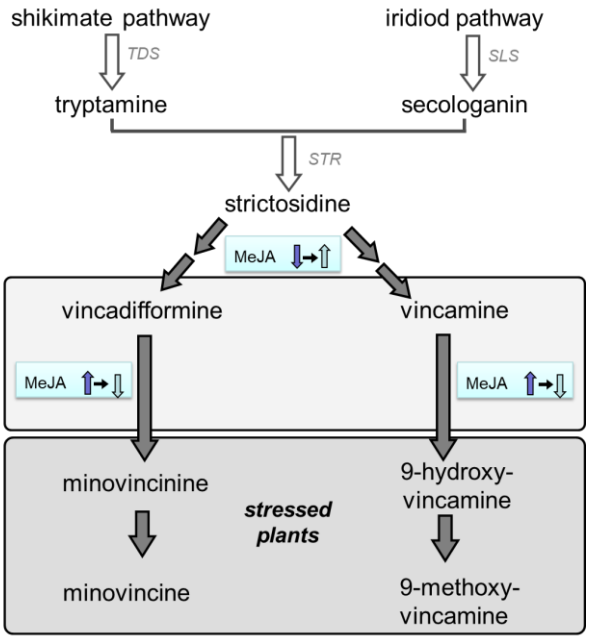


Figure 4-18b: Effects of MeJA in the conversion of major indole alkaloids in *V. minor* as outlined in Figure 4-18 (SLS, secologanin synthase; STR, strictosidine synthase; TDC, tryptophan de-carboxylase). ⇩→⇧: the decrease in vincamine and vincadifformine concentrations at 8 days followed by increase after 14 days. ⇧→⇩: the increase in minovincine, minovincinine and methoxyvincamine at 8 days followed by massive decrease at 14 days.

The complexity of the signal transducing effects of jasmonic acid is vividly demonstrated by the putative contradictory findings in *C. roseus*. Whereas in hairy root cultures it leads to a significant increase in the content of indole alkaloids and to a shift in their composition (Rijhwani and Shanks, 1998; Rodriguez *et al.*, 2003), it seems to have no effect on the indole alkaloid accumulation in mature *C. roseus* leaves (Pan *et al.*, 2010). Moreover, the response of developing seedlings of *C. roseus* to MeJA varies strongly (Aerts *et al.*, 1994). Schluttenhofer et al. (2014) tried to explain these contradictions by the involvement of the WRKY transcription factors, which are known to be key regulators of many syndromes in plants, including the processes induced by drought or salt stress, and also the responses related to the jasmonate signal transduction (Phukan *et al.*, 2016). The authors showed that the MeJA related induction of the strictosidine synthase, the key enzyme of indole alkaloid biosynthesis, is correlated with corresponding changes in the expression of WRKYs. However, as the WRKYs also regulate various stress-related temporary downstream steps in modifications of the main alkaloidal structures, the diverse responses may interfere, resulting in a complex physiological situation (Schluttenhofer *et al.*, 2014). Many further approaches are required to elucidate the observed changes in the pattern of indole alkaloids in *V. minor*.

Hydrogen peroxide (H_2O_2) is a stress-related by-product of cellular metabolism. In plants, it is produced in large amounts chloroplasts whilst photorespiration and in peroxisomes in the course of the ß-oxidation of fatty acids (Slesak *et al.*, 2007; Mubarakshina *et al.*, 2010; Corpas, 2015). Moreover, hydrogen peroxide acts as an important substrate for many peroxidases, e.g., those involved in the lignin biosynthesis. Apart from these processes, in which relatively large concentrations of H_2O_2 are involved, it also represents an important signal molecule. Growing evidence suggests that H_2O_2, sometimes in combination with other reactive oxygen species (ROS), plays a versatile role in eliciting plant defense and various developmental processes (Quan *et al.*, 2008; Saxena *et al.*, 2016). In this sense, ROS regulate cell proliferation and differentiation, programmed cell death, seed germination, root hair growth and pollen tube development, senescence, etc (Singh *et al.*, 2016). In principle, H_2O_2 accumulation is maintained at a very low level because of the existence of an antioxidant system in plants, which are responsible for eliminating the excess of H_2O_2, and thereby keeping the level of H_2O_2 in a dynamic balance. This "redox homeostasis" is achieved by various enzymes and several chemical scavengers. The enzymatic antioxidant system includes ascorbate peroxidases, superoxide dismutases, catalases, glutathione peroxidases, and glutathione reductases. The most abundant antioxidant molecules are tocopherol, ascorbic acid, glutathione and many secondary metabolites, including MIAs (Matsuura *et al.*, 2014).

Various studies have shown that the exogenous application of H_2O_2 to plants caused a rapid production of endogenous H_2O_2 and thereby to a destruction of the homeostatic redox status (Allan and Fluhr, 1997; Zhao *et al.*, 2001a; Tang *et al.*, 2009) and thus, to oxidative stress[5].

In response to many abiotic stress situations, one of the major effects is the generation of an "over-reduced status" in the chloroplasts (Wilhelm and Selmar, 2011; Kleinwächter and Selmar, 2014). The stress-related stomata closure massively diminishes the CO_2-influx into the leaves. In consequence, far less reduction equivalents ($NADPH+H^+$) are consumed via the Calvin cycle. Although the various energy dissipating mechanisms are up-regulated, the reduction status of the chloroplasts increases massively (Selmar and Kleinwächter, 2013; Kleinwächter and Selmar, 2015). As result, electrons from the photosynthetic electron transport chain will directly be conveyed to oxygen, generating superoxide radicals, which subsequently have to be detoxified by superoxide dismutase (Selmar *et al.*, 2017). Indeed, the major share of the H_2O_2 is detoxified by ascorbate peroxidase, nevertheless, the overall content of ROS

[5] Oxidative stress is the condition in which the production of reactive species exceeds antioxidant defenses thereby disturbing the redox balance.

strongly is enhanced (Matsuura *et al.*, 2014). In addition, H_2O_2 is generated in many other cell organelles by various reactions (Slesak *et al.*, 2007; Mubarakshina *et al.*, 2010; Corpas, 2015). Accordingly, oxidative stress, i.e., the generation of ROS, is a common outcome of biotic and abiotic stresses, and may lead to several responses, including antioxidant systems activation and secondary metabolites elicitation, processes that may be interconnected for successful protective responses Figure 5-6. In summary, H_2O_2 represents a versatile molecule already acts as an important signal molecule at normal levels. However, due to its massive increase under abiotic or biotic stress conditions, it induces oxidative stress.

Due to its unique properties, i.e., its stability, diffusibility and lesser reactivity, H_2O_2 should be differentiated from the rest of the ROS molecules (Quan *et al.*, 2008), predestining it as a good signaling molecule. Accordingly, in plants, non-toxic levels of H_2O_2 act as key factor in intracellular signalling, e.g., to regulate numerous physiological and biological processes and their regulation and triggering (Sena and Chandel, 2012), such as growth, hypoxic signal transduction, autophagy, and stem cell proliferation and differentiation (Holmström and Finkel, 2014; Reczek and Chandel, 2015). Moreover, H_2O_2 is involved in the elicitation of biotic and abiotic stress responses (Reczek and Chandel, 2015; Mittler, 2017) and interferes with various hormonal signaling pathways to regulate plant growth and stress tolerance.

One of the best-characterized mechanisms, by which H_2O_2 act as signal molecules is the oxidation of cysteine residues within redox-sensitive proteins (D'Autréaux and Toledano, 2007). At toxic concentrations H_2O_2 is involved in programmed cell death (Dat *et al.*, 2003): when large amounts of H_2O_2 react with metal cations (Fe^{2+} or Cu^{+}), in the course of this Fenton reaction high concentrations of the extremely reactive hydroxyl radical ($OH^{\bullet}$) are formed, which cause irreversible oxidative damage. Accordingly, the particular signaling effect of H_2O_2 or its typical chemical action depends on its concentration (Figure 5-6).

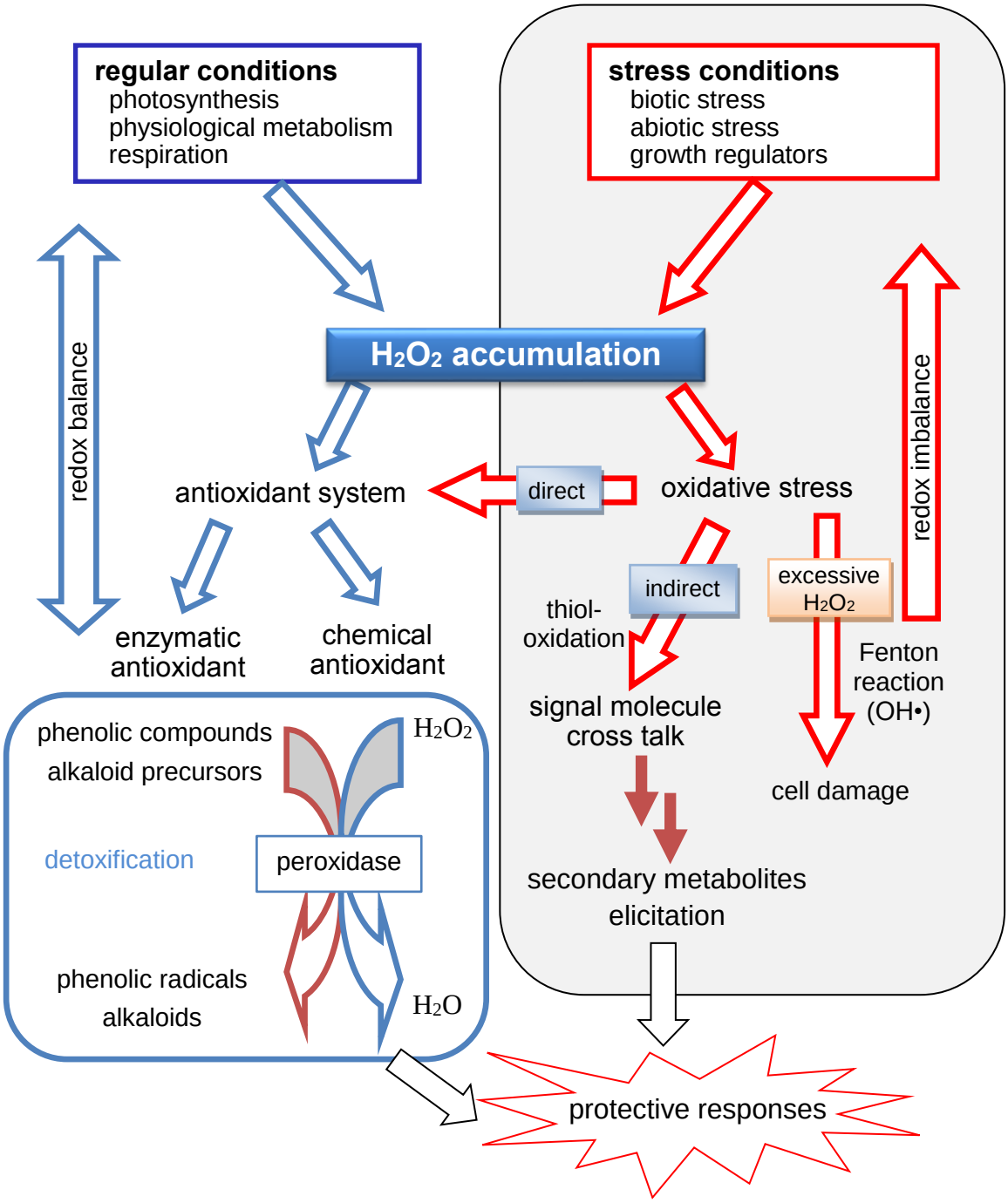


Figure 5- 6: Involvement of H_2O_2 in regular metabolism and in stress responses. Excess hydrogen peroxide generates oxidative stress by producing hydroxyl radicals. Moreover, H_2O_2 is also acting indirectly as a second messenger in responses to various stress situations. In addition, hydrogen peroxide is interfering with several other signal molecules (crosstalk). In consequence, H_2O_2 is part of the induction and regulation system to protect plants from the stresses and in regulating plant growth and development. In general, the concentration of H_2O_2 is maintained at a basic level by the concomitant action of a series of antioxidant systems.

Zhao et al. (2001a) reported that both, exogenous H_2O_2 treatment and elicitor-induced H_2O_2 overproduction are associated with related changes in alkaloid production. The author suggested a close relationship between the oxidative burst and indole alkaloid biosynthesis in

elicited *C. roseus* cell cultures. Up to now, there are only a few reports, in which a direct impact of oxidative stress on the concentration of MIAs are displayed. In this context, Tang et al. (2009), documented an identical time course of the vinblastine and H_2O_2 in seedlings of *C. roseus* in response to oxidative stress. In the same sense, several reports mentioned that in stressed *C. roseus*, the increase in the concentration of vinblastine and serpentine correlates with that of H_2O_2 and an enhancement of the peroxidase activity present in vacuoles of mesophyll cells (Knobloch *et al.*, 1982; Loyola-Vargas *et al.*, 1992; Sottomayor *et al.*, 2004). From these coherences, it was postulated that the peroxidase catalyzed the oxidation of ajmalicine into serpentine as well as the oxidative coupling of vindoline and catharanthine are part of an antioxidative system responsible for the detoxification of H_2O_2 under stress conditions (Sottomayor *et al.*, 2004). Analogously, the indole alkaloids in *Camptotheca acuminata* are also assessed as antioxidative compounds, based on the consumption of H_2O_2 when camptothecin is oxidatively converted to 10-hydroxycamptothecin in response to hydrogen peroxide elicitation (Pi *et al.*, 2010). Moreover, in the hairy root of *V. minor*, H_2O_2 caused a significant enhancement of total alkaloid content (Verma *et al.*, 2014b). In conclusion, it is argued that the indole alkaloids in combination with the vacuolar peroxidases represent an effective system to detoxify stress related H_2O_2 in levels in green plants.

As mentioned above, H_2O_2 may act directly as a substrate in the oxidative system and thereby modulate MIAs via peroxidase catalyzed reaction (Zhao *et al.*, 2001b; Sottomayor *et al.*, 2004; Tang *et al.*, 2009) or it could act indirectly via impacting the signal transduction. In the latter case, the signal molecule may change the concentration and spectrum of MIAs by inducing enzymes involved in biosynthesis, allocation or modification. Moreover, it may interfere with other signal molecules, such as JA, SA, ethylene, Ca^{++}, NO etc. (for review see (Dat *et al.*, 2000; Saxena *et al.*, 2016) and thereby alter signal transduction. In this sense, H_2O_2 accumulated in response to high light in tobacco plant up-regulate genes involved in the hypersensitive response, vesicular transport, posttranscriptional processes, biosynthesis of ethylene and jasmonic acid, proteolysis, mitochondrial metabolism, and cell death, and was accompanied by a very rapid up-regulation of several signal transduction components (Vandenabeele *et al.*, 2003).

With respect to its physicochemical properties, H_2O_2 is considered as an ideal signaling molecule: it is the most stable ROS, reveals a selective reactivity, and a high diffusibility. Moreover, it interacts with thiol-containing proteins. In consequence, it activates different

signaling pathways as well as various transcription factors that in turn regulate gene expression and cell-cycle processes (Møller *et al.*, 2007; Slesak *et al.*, 2007).

A major problem in the understanding of the complex action of H_2O_2 is related to spatial compartmentation. On the one hand, the impact of H_2O_2 is described to related to its site of origin, i.e., if produced in peroxisomes, it induces transcription of processes related to protein repair responses, while H_2O_2 production in chloroplasts leads to early signal responses, including transcription factors and biosynthetic genes involved in production of secondary messengers (Sewelam *et al.*, 2014). On the other hand, hydrogen peroxide is highly diffusible. Accordingly, the balance between hydrogen peroxide production and its removal determines its actual concentration and thus the mode and extent of its action, i.e., the extent and efficiency of signaling processes and/or that of oxidative damage (Møller *et al.*, 2007).

The application of hydrogen peroxide resulted in a similar change of the MIAs pattern as the treatment with MeJA, i.e., the conversion of vincamine to 9-methoxyvincamine and of vincadifformine to minovincinine and minovincine (Figure 4-18c). However, in the case of H_2O_2 treatment, in the beginning, the concentration of both putative precursors (vincamine and vincadifformine) increased before they - concomitantly with the increase of the products (9-methoxvincamine, minovincinine, and minovincine) - decreased again (Figure 4-18c). Obviously, in addition to the impact of jasmonic acid, the elicitation by H_2O_2 resulted in *V. minor* first in an initial increase of MIAs biosynthesis leaves, before the same conversions are induced. On the first glance, these modifications could be related to peroxidase catalyzed reactions with H_2O_2 as outlined above for serpentine and vinblastine. However, according to the results from the concomitant application of signal transducers and enzyme inhibitors, a direct contribution of H_2O_2 in the conversion of vincamine and vincadifformine to 9-

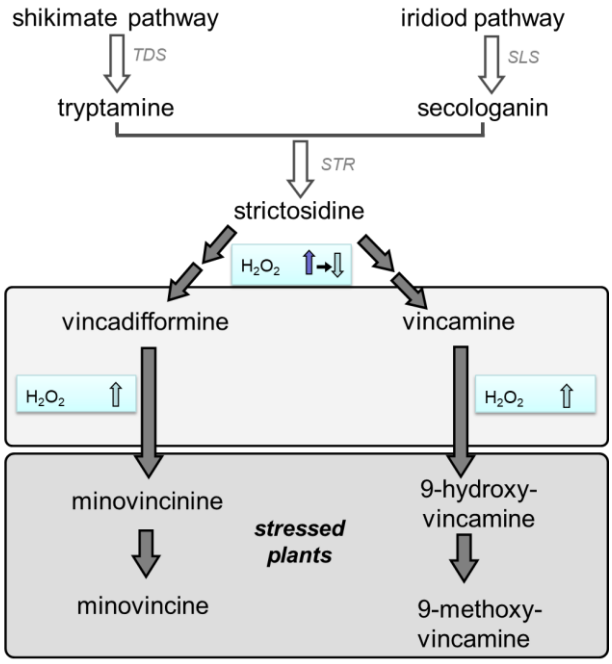


Figure 4-18c: Effects of H_2O_2 in the accumulation and modification of indole alkaloids in *V. minor* as outlined in Figure 4-18. ⇧→⇩: the transient increase in vincamine and vincadifformine concentrations till 8 days followed by massive decrease after 14 days. ⇧: the increase in minovincine, minovincinine and methoxyvincamine at 14 days.

hydroxyvincamine and to minovincine (Appendix, Figure A-71) could be ruled out: the simultaneous application of H_2O_2 with naproxen[6] resulted in a complete inhibition of the conversion. As this inhibitor does not affect peroxidases, a contribution of peroxidases is very unlikely. Moreover, as outlined below in detail, the outcome of resveratrol and naproxen application indicates the involvement of cytochrome P450 enzymes.

These results suggest that indole alkaloids may be involved in plant defense, i.e., against pathogens or to repel herbivores. The animal attack is frequently associated with wounding, which in turn depends on the mode of herbivory, e.g., by chewing or sucking actions. The related differences in tissue destruction induce various modifications of a plant's wound responses (Wu and Baldwin, 2009). MeJA, ethylene (ET), and reactive oxygen species (ROS e.g. H_2O_2), which are involved in activation of the various wounding-responsive genes, mediate the signaling of the wounding response and pathogenesis-related genes (Jacobo-Velázquez *et al.*, 2015). According to Jacobo-Velázquez et al. (2015), ROS (e.g. H_2O_2) play a key role as signaling molecules for the wound-induced activation of the primary and secondary metabolism, whereas ET and MeJA are thought to be essential for the modulation of the ROS levels. In this sense, an initial response to wounding, ROS, and ET are simultaneously produced. After that, the later one, i.e., ET, induces the biosynthesis of JA, which, in turn, plays a key role in modulating the ROS levels, especially that of H_2O_2, afterward regulates the expressions of numerous genes involved in the wounding responses (Jacobo-Velázquez *et al.*, 2015). Moreover, hydrogen peroxide modulates the intensity of the various signaling pathways (Ma *et al.*, 2015), resulting in a complex so-called cross-talk of the different signal transducers (Saxena *et al.*, 2016).

In consequence, like MeJA, also H_2O_2 can be utilized to modulate the pattern of MIAs in *V. minor* and thus to alter the pharmacological properties of corresponding plant extracts. Since hydrogen peroxide also induces the generation of MeJA, e.g., via lipid peroxidation, a strict differentiation between the effects of both signal transducers could not be made. However, the time course of the alterations is quite different.

[6] In animals, naproxen inhibits cyclo-oxygenase. In Plants, endogenous jasmonate production is inhibited by the inhibition of alleneoxide synthetase, a cytochrome P450 enzymes (Pan *et al.,* 1998). Naproxene does not inhibit peroxidases (Tam *et al.,* 1995).

The effect of inhibitors of oxidative enzymes

As mentioned above, the conversion of vincamine and vincadifformine are thought to be catalyzed by cytochrome P450[7] enzymes (CYPs), not least because of the similarities to related modifications of the indole alkaloids elucidated for *C. roseus*, (Figure 5-7; (Rodriguez *et al.*, 2003; Giddings *et al.*, 2011).

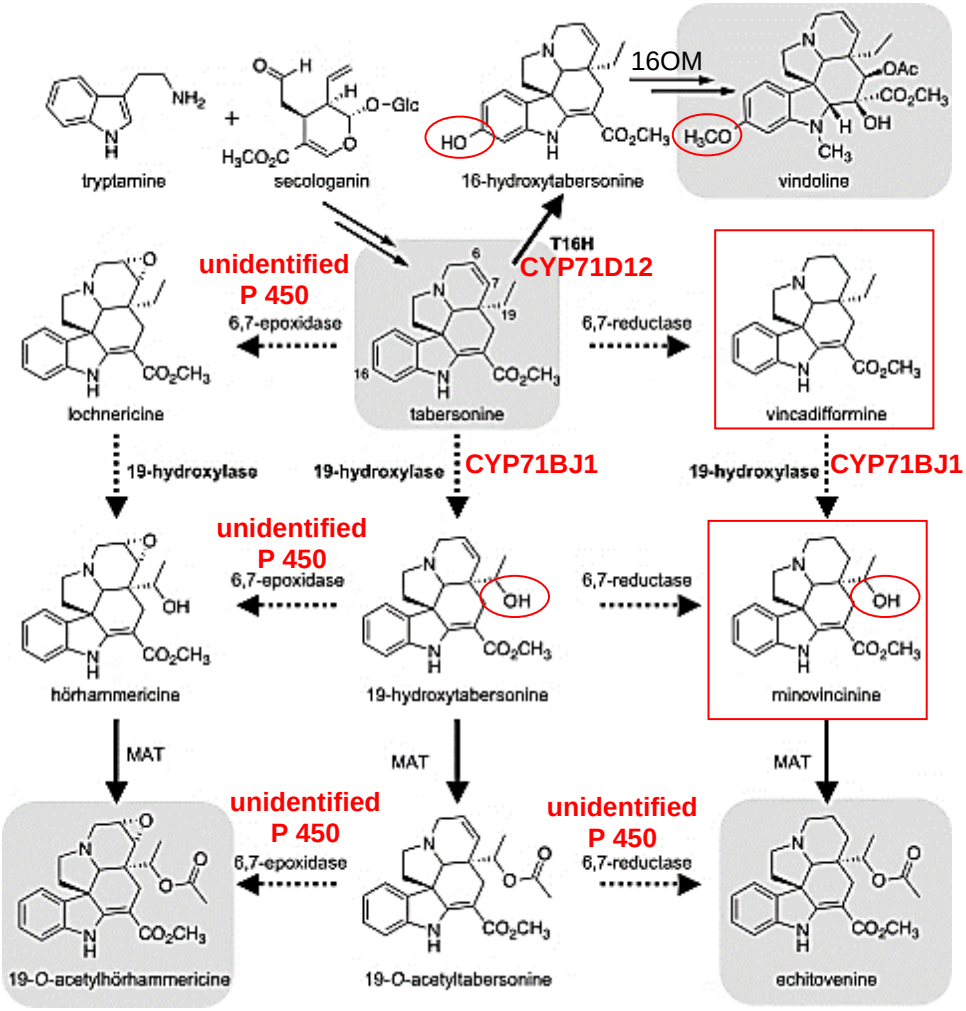


Figure 5- 7: Involvement of cytochrome P450 enzymes in the biosynthesis of tabersonine-derived alkaloids in *C. roseus*. Most of the biosynthetic steps in the roots are still uncharacterized at the genetic level and are represented by *dashed arrows*. However, most of the reactions are known to be catalyzed by cytochrome P450 enzymes (Hotze *et al.*, 1995; Irmler *et al.*, 2000; Collu *et al.*, 2001; Facchini and Luca, 2008; Giddings *et al.*, 2011).

[7] In general, CYPs catalyze regio- and stereoselective oxidative attack of non-activated carbons at physiological conditions (Akashi *et al.*, 1999). They are responsible for various general reactions in plant primary as well as secondary metabolism, e.g., hydroxylations (Rodriguez *et al.*, 2003; Giddings *et al.*, 2011). However, few enzymes exhibit less common unusual activities (Mizutani and Sato 2011; Guengerich and Munro 2013) including successive oxidation of a single carbon (Helliwell *et al.* 2001; Carelli *et al.*, 2011), rearrangement of carbon skeletons (Akashi *et al.*, 1999).

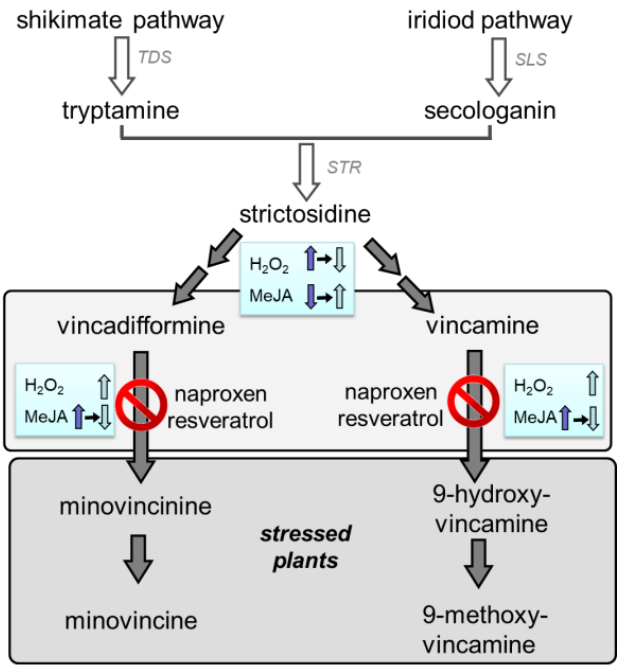
In the aerial parts of *C. roseus*, the alkaloid tabersonine is transformed into vindoline, whereas in the roots it is converted to 19-O-acetylhörhammericine (see Figure 5-7; Laflamme *et al.*, 2001; O'Connor and Maresh, 2006). Both, the hydroxylation at the 16-position of tabersonine, which ultimately leads to vindoline, and the conversion of tabersonine to form lochnericine are catalyzed by P450-dependent enzymes (Furuya *et al.*, 1992; Shanks *et al.*, 1998). Moreover, tabersonine is converted to 19-hydroxytabersonine by the action of a P450-dependent 19-hydroxylase (Morgan and Shanks, 1999; Giddings *et al.*, 2011; Figure 5-7). Previous studies reported that many of the cytochrome P-450 enzymes involved in alkaloid biosynthesis are inducible by MeJA (Pauli and Kutchan, 1998; Morgan and Shanks, 1999; Rodriguez *et al.*, 2003; Giddings *et al.*, 2011).

To determine the enzyme activities of P450 is problematic, it requires the close collaboration with an appropriate NADP reductase and the availability of the specific substrate. Thus, unfortunately, a reliable and reproducible quantification of the enzyme activity has not yet been developed. An alternative approach to verify the involvement of a CYP is based on the reduction of their enzyme activity via the application of corresponding inhibitors or competitive substrates, respectively. The use of oxygenase inhibitors has proved useful for the study of the tabersonine branchpoint (Morgan, Shanks 1999) and catharanthine biosynthesis (Morgan and Shanks, 1999; Guo *et al.*, 2011; Guo *et al.*, 2013) in *C. roseus*. Similar studies have examined the biosynthetic pathways of taxol (Srinivasan *et al.*, 1996), brassinolides (Winter *et al.*, 1997) and benzylisoquinoline alkaloids (Sugimoto *et al.*, 1997). In contrast, in hairy root cultures of *V. minor*, oxygenase inhibitors (e.g. naproxen) had no effect, neither on total alkaloid nor on vincamine content. However, the combination of naproxen with H_2O_2 increases the total alkaloid content (Verma *et al.*, 2014b). In plants, naproxen and other nonsteroidal-anti-inflammatory drugs are described to hampers the endogenous jasmonate production by the inhibition of the alleneoxide synthetase, a typical cytochrome P450 enzyme catalyzing the key reaction in the biosynthesis of jasmonic acid (Staswick *et al.*, 1991; Nojiri *et al.*, 1996; Pan *et al.*, 1998; Froehlich *et al.*, 2001; Hu *et al.*, 2010). In conclusion, naproxen is known to be a substrate for various cytochrome P450 enzymes (Miners *et al.*, 1996; Tracy *et al.*, 1997). Accordingly, it was employed in this study to inhibit the enzymes putatively responsible for the stress-related modifications of MIAs in *V. minor*.

Resveratrol, a stilbene, which is well known for its antioxidative and health protective properties is also described to act as competitive substrate for methyltransferases as well as for P450 enzymes and thereby inhibit the turnover of the genuine substrates of these enzymes (e.g. P450 hydroxylase; Piver *et al.*, 2001; Martínez-Márquez *et al.*, 2016). Accordingly, apart from naproxen, also this stilbene was used to modify the biosynthesis of MIAs in *V. minor*.

When inhibitors for P450 enzymes, either resveratrol or naproxen, respectively, were applied together with MeJA, the common MeJA induced conversion of vincadifformine and vincamine does not occur. This fascinating effect could be caused either indirectly by a suppression of MeJA related signaling pathway or directly by an inhibition of the enzymes responsible for the conversion of vincamine and vincadifformine, respectively. Up to now, no data on the inhibition of the signal transduction downstream jasmonic acid are known. Yet, one key reaction within the synthesis of the jasmonic acid itself is catalyzed also by a P450 enzyme, i.e., the allene oxide synthase (Pan *et al.*, 1998). However, as high amounts of MeJA had been applied exogenously, a putative inhibition of endogenous biosynthesis of jasmonic acid should not influence the corresponding induction.

In addition to the inhibition by naproxen also resveratrol, which is described as a competitive substrate for cytochrome P450 enzymes, also blocks the conversion of vincadifformine to minovincine as well as the hydroxylation of vincamine. Accordingly, it could be assumed that these reactions indeed are catalyzed by P450 enzymes. This assumption strongly is supported by the fact that *in C. roseus* the congruent reactions are known to be catalyzed by such cytochromes (Figure 5-7; 4-21d). The further investigation of this fascinating issue corresponds to a promising and auspicious scientific approach.



Salicylic acid (SA) is further important plant growth regulator that modulates various processes in plant growth and development (Pancheva and Popova, 1998; Scott *et al.*, 2004), and leaf senescence (Abreu and Munné-Bosch, 2007). However, the predominant significance of SA is related to systemic acquired resistance; i.e., it mediates plant defense against pathogens and some sucking insects (Walling, 2009; Kästner *et al.*, 2014), and increases systemically the resistance and plant fitness (Traw *et al.*, 2007). Accordingly, SA represents an important factor in the interaction of plants with their environment.

In contrast to the impact of MeJA and H_2O_2 on indole alkaloid modifications, SA treatment resulted in an initial and transient increase of the vincamine and vincadifformine accumulation in the leaves. A similar response was observed by Pan et al. (2010), who reported that exogenously applied SA to *C. roseus* also caused a transient increase in indole alkaloid content.

SA does not induce the conversion of vincamine to methoxyvincamine and of vincadifformine to minovincine and minovincinine, respectively. When SA has applied again after four days, the content of vincadifformine decreased significantly. It appears that at least high concentrations of salicylic acid resulted in an inhibition of various reactions involved in the synthesis of MIAs, or in an induction of their catabolism. A related finding was reported by Guo et al. (2013), who showed that salicylic acid does not act as elicitor for tabersonine biosynthesis. However, when SA is applied to seedlings or suspension cultures, respectively, the content of alkaloid increased (Godoy-Hernández and Loyola-Vargas, 1997; El-Sayed and Verpoorte, 2004). In contrast, other investigation could not verify such impact of SA on the indole alkaloid metabolism: the application of SA to suspension cultures of *C. roseus* (Guo *et al.*, 2013) as well to seedling of *C. roseus* (Aerts *et al.*, 1996) and to leaves of *Psychotria brachyceras* (Gregianini *et al.*, 2004) had no effect on the concentration of indole alkaloids. Gregianini et al. (2004) tried to explain these putative contradictions by suggesting that the significance of indole alkaloids might not be restricted to general interaction with pathogens but might mainly be assigned to general wound responses and to protection against UV light, which probably is mediated by jasmonic acid. The corresponding multifarious interactions between the different growth regulators might explain the inconsistent findings outlined.

As mentioned above, SA is mainly associated with the establishment of systemic acquired resistance and its levels increase after pathogen infection (Menke *et al.*, 1999), but also in processes related to wounding and to the resistance against insects. As highlighted above, various evidence indicates that the signal pathways related to SA are not linear, but are integrated through a network of cross-talking connections to coordinate various different

responses. In this sense, especially the substantial communication between the JA and SA signal transductions seem to be vital. Nevertheless, JA and SA signal cascades might activate different sets of plant defense genes (Thomma *et al.*, 1998) or in some cases, they even act antagonistically (Felton *et al.*, 1999; Leon-Reyes *et al.*, 2010). Exogenous application of SA or its derivatives, such as aspirin, blocks both JA biosynthesis and thus the effect of JA in wound signalling (Doherty *et al.*, 1988; Pena-Cortés *et al.*, 1993; Doares *et al.*, 1995; Kunkel and Brooks, 2002; Leon-Reyes *et al.*, 2010). Thus, depending on the type of induction, there might exist different control pathways or partial response superposition (Baron and Zambryski, 1995; McConn *et al.*, 1997; Maleck, 1999; Pieterse *et al.*, 2012).

According to the results presented in this thesis, the exogenous application of SA might act as antagonist for the endogenous JA signal by inhibiting the allene oxide synthase (AOS) (Sivasankar *et al.*, 2000; Norton *et al.*, 2007), which catalyzes the first step in the biosynthesis of jasmonic acid. In addition, experiments with *Arabidopsis* revealed that JA-responsive marker genes are highly sensitive to suppression by exogenous application of SA. This suggests that the significance and the accumulation of indole alkaloids might not be restricted to interaction with pathogens, but might also be assigned to general wound responses, which probably might be mediated by JA. In contrast, Mur et al. (2006) reported that in *Arabidopsis*, a treatment with low concentrations of JA and SA resulted in a synergistic effect on the JA- and SA-responsive genes. These different observations strongly suggest further research to study the interactive effect of MeJA and SA, especially at low and different concentrations on the in the indole alkaloid accumulation and modulation in *V. minor* plants.

The complex interaction of signal molecules

Elicitation is vastly complex because of an almost infinite number of intertwined events. Additionally, all these events might differ with respect to temporary and spatial variations. Moreover, these processes strongly are impacted by the environment (Shakya *et al.*, 2017; Figure 5-8). Furthermore, all these alterations depend on the physiological and developmental stage of the plant, their growth cycle and nutritional status, as well as the specificity and concentration of elicitors, which, in turn, massively influence the final responses (Shakya *et al.*, 2017). Thus, it is nearly impossible to establish a universal and absolute model, which comprises all elicitation mechanisms. Nevertheless, to increase the basic understanding of these processes, corresponding schemes are required, in which the particular components, such as calcium flux, a burst of reactive oxygen species (ROS), and phosphorylation by mitogen-activated protein kinase (MAPK) are outlined (Seybold *et al.*, 2014). As mentioned above, these

reactions might initiate further signaling events, i.e., the activation of transcription factors and the induction of plant secondary metabolism (Trewavas and Malhó, 1998; Naoumkina *et al.*, 2008; Schluttenhofer and Yuan, 2015; Pan *et al.*, 2016).

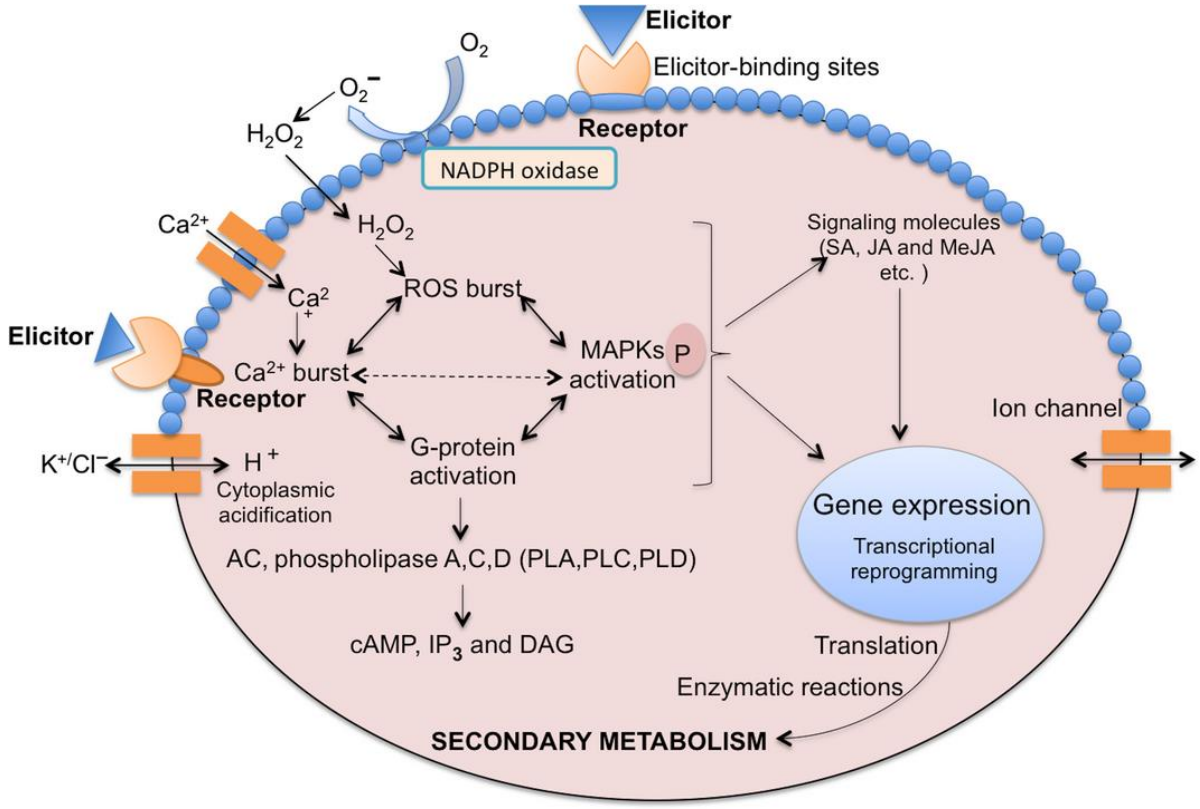


Figure 5- 8: The molecular mechanism of elicitation at the cellular level: Recognition of elicitors by plasma membrane-bound receptors results in ion fluxes, Ca^{2+} burst, cytoplasmic acidification, ROS burst, activation of NADPH oxidases, of mitogen-activated protein kinases (MAPKs) and of G-proteins. These events interfere with other signaling pathways, e.g., those involving salicylic acid, jasmonic acid, cAMP, which in turn might activate transcription factors and thus gene expression, which modulates – apart from many other processes – also the secondary metabolism (Shakya *et al.*, 2017).

In conclusion, after the signaling molecules are produced, various signal transduction mechanisms are activated and the primary, as well as the secondary metabolisms, are modulated in the stressed plants. With respect to defense reactions, a wide array of processes might be induced. After being infected by a pathogen, the major defense reaction is the so-called hypersensitive response; i.e., the cells die. Due to the ongoing de-compartmentation, tanning reaction causes the typical browning processes, which hamper most infections. In the areas around the center of the infection, i.e., the cells surrounding the necrotic tissue, further defense reactions are generally induced. When focusing on the underlying mechanisms, it has to be considered that in principle there are two different basic strategies (VanEtten *et al.*, 1994), either the defense substances are constitutively present in the plants or they are synthesized in response to the infection. In the first case, they are denoted as phytoanticipins, the latter ones are classified as phytoalexins.

When phytoanticipins are constitutive accumulated in the plants, they either might be already active, like alkaloids, saponins or various phenolic compounds, or they are activated postmortal from pre-existing precursors like polyphenols, cyanogenic glucosides or glucosinolates. When the integrity of the cell is destroyed, i.e., due to insects feeding or penetration by fungal hyphae, the precursors come into contact with their degrading enzymes. In this sense, the hypersensitive reaction is due to the enzymatic oxidation of polyphenols and cyanogenic glucosides or glucosinolates are hydrolyzed and toxic aglycones are released. The latter process frequently is denoted as a biological bomb (Matile, 1980; Tomas-Barberan and Gil, 2008; Iriti and Faoro, 2009). In contrast to the phytoanticipins, the phytoalexins are synthesized *de novo* from primary metabolites in response to the pathogens attack (Jeandet, 2015; Pusztahelyi *et al.*, 2015), or upon UV radiation (Marti *et al.*, 2014).

It is worth mentioning that the processes responsible for the induction of both defense strategies, the hypersensitive reaction and the synthesis of phytoalexins are more or less identical: in the first phase of host-pathogen interaction, due to the cellulolytic enzymes released from the pathogens, fragments of the plant cell wall are liberated. These signal molecules denoted as elicitors, are recognized by corresponding receptors (Figure 5-8), and the various signal transduction cascades are induced. Apart from many other events, also glucanases, chitinases, and other hydrolases are produced, which degrade the cell walls of the attacking pathogens. The corresponding fragments also operate as elicitors and the entire elicitation process is enhanced and accelerated. Interestingly, the decision whether the hypersensitive reaction is initiated or the cells remain alive and the other protective reactions are induced, depends on the concentration on elicitors and on the velocity of its generation (Fritig *et al.*, 1987). In the same sense, the subsequent generation and accumulation of reactive oxygen species (ROS) and of nitric oxide (NO) determines the mode and outcome of the defense mechanism. Accordingly, active oxygen species might be part of the hypersensitive response, or they are responsible directly for the death of the cells from the host as well as from the pathogens. Moreover, in lower concentrations, they might act as secondary messenger and thus are part of the signal transduction chains. These coherences clearly display the tremendous complexity of the entire issue. Accordingly, a comprehensive conception of the elicitation of secondary metabolism by various signal transducers does not only require – as outline above - the consideration of the specific developmental and physiological status of the plants, but also the concentration of the signal molecule applied.

The situation is even more complex when the multifarious interactions of the different phytohormones and numerous growth regulators are considered. Up to now, we are far away to understand and conceive all these reciprocal interferences, frequently denoted as cross-talk. In

this context, the various interaction of jasmonic acid (JA) and salicylic acid are a fascinating example, which vividly displays the entire complexity of this issue (Pieterse *et al.*, 2009). As outlined in Figure 5-9, in the course of a typical host-pathogen interaction, SA-dependent resistance against biotrophic pathogens and JA-dependent defense against necrotrophic pathogens and herbivory have been documented (Bostock, 2005; Stout *et al.*, 2006). For example, induction of the SA pathway in *Arabidopsis* by the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* strongly is suppressed by JA-mediated defense reactions that were activated upon feeding by caterpillars of white *Pieris rapae* (Koornneef *et al.*, 2008). Although many reports describe an antagonistic interaction between SA- and JA-dependent signaling, also many synergistic interactions have been described as well (Mur *et al.*, 2006). In this sense, the treatment with low concentrations of JA and SA resulted in *Arabidopsis* in a synergistic effect on the activation of JA- and SA-responsive genes. However, at higher concentrations the related effects were antagonistic. This, in turn, demonstrates that the outcome of the SA-JA interactions strongly dependents on the actual concentration of each growth regulator (Mur *et al.*, 2006). Since their concentrations also are impacted by the various anabolic as well catabolic reactions, Koornneef et al. (2008), outlined that – apart from the actual concentration - timing and sequence of initiation of SA and JA signaling are very important factors for the outcome of the SA-JA signal interaction.

The complex interaction between SA and JA also plays a major role in the induction of systemic acquired resistance (SAR): SA, as well as JA or its derivatives, are translocated as within the plant from one organ to another (Farmer, 2001; Durrant and Dong, 2004; Attaran *et al.*, 2009). Yet, this relocation of growth regulators causes a further level of complexity, since also nonpathogenic microorganisms existing on the leaves or in the rhizosphere might induce SAR and the related translocation of growth factors (Attaran *et al.*, 2009; Beneduzi *et al.*, 2012). In this context, beneficial soil-borne microorganisms, such as mycorrhizal fungi and plant growth promoting rhizobacteria, are of special interest. In analogy to SAR, the related induced reactions frequently are denoted as induced systemic resistance (ISR; Figure 5-9 ; (van Loon *et al.*, 1998; Pozo and Azcón-Aguilar, 2007). This means that the interaction with various beneficial microorganisms could cause a systemic activation of several responses related to the various defense reactions. In this sense, the growth of several rhizobacteria triggers the synthesis of SA and thus the SA-dependent SAR pathway. In addition, at least in *Arabidopsis thaliana*, the induced systemic resistance (ISR) is mediated by a SA-independent pathway, which is triggered by jasmonic acid (JA) and ethylene. A similar mechanism is described for the induction of defense reactions due to non-pathogenic microorganisms growing on leaves; (Bargabus *et al.*, 2002). The increase of resistance due to the induction of ISR frequently is denoted as "priming" (Heil, 2001; Choudhary *et al.*, 2007). Whereas SAR is predominantly effective against

biotrophic pathogens that are sensitive to SA-induced defense mechanisms, ISR was shown to be effective mostly against pathogens and insects that are sensitive to the primarily JA- and ET-induced defense reactions (Ton *et al.*, 2002; van Oosten *et al.*, 2008). Combination of ISR and SAR can increase protection against pathogens that are resisted through both pathways besides extended protection to a broader spectrum of pathogens than ISR or SAR alone (Choudhary *et al.*, 2007).

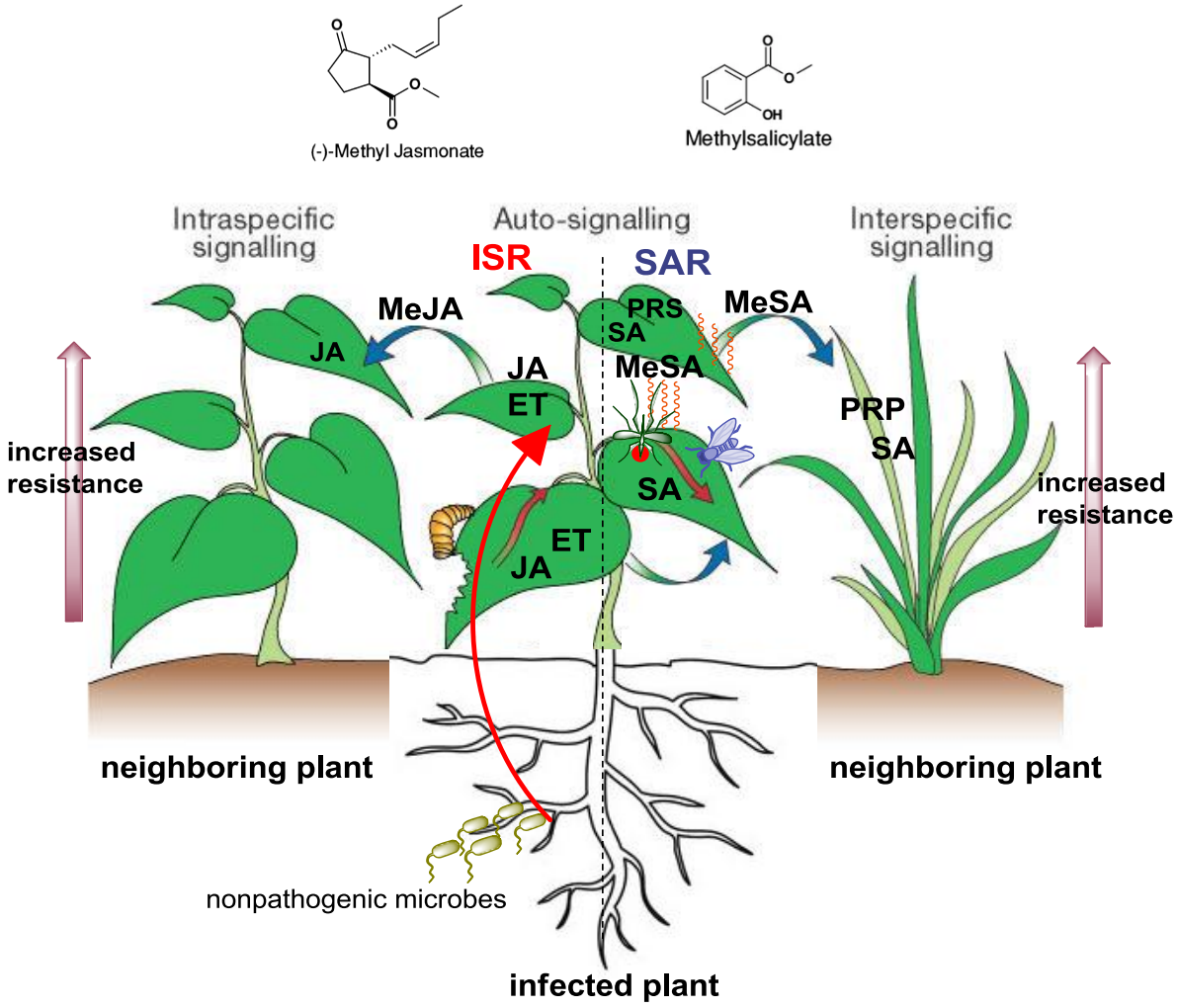


Figure 5- 9: Interactions and modifications of the SA and JA depending signaling.
Whereas the responses related to the systemic acquired resistance (SAR) are caused by pathogen or herbivore attack, those involved in induced systemic resistance (ISR) are triggered by non-pathogenic microorganisms, predominately by microbes growing in the rhizosphere. Both phenomena, which mandatorily involve SA and JA signaling, lead to an increased resistance against future attacks by pathogens or herbivores. A corresponding enhanced defense status could also be induced by transferring the airborne signaling molecule methyl jasmonate (MeJA) and methyl salicylate (MeSA) from infected organs of one plant to distant leaves either of the same plant or the same species respectively, or to plants of another species, growing in the near vicinity. This figure is modified from (Farmer, 2001; Taiz and Zeiger, 2010; Pieterse *et al.*, 2009; Pieterse *et al.*, 2012).

Additionally, the growth factors can be readily modified into derivatives revealing altered biological activity and physicochemical properties, opening the door for another layer of

regulation (Pieterse *et al.*, 2009). The best examples for such derivatives are the volatile methyl ester of SA and JA, which are proposed to be relevant for long-distance signalling from infected or injured organs to distant leaves either of the same plant or of individuals growing in the near vicinity (Farmer, 2001; Attaran *et al.*, 2009; Tamogami *et al.*, 2012). In this sense, methyl salicylate is produced by infected plants increases the resistance of neighboring, uninfected tobacco plants, e.g., by inducing the expression of the defense gene *PR1* in uninfected plants (Shulaev *et al.*, 1997). Meanwhile, further compounds, which act as airborne plant-to-plant signals, have been identified, e.g., MeJA is liberated from infected big sagebrush plants (*Artemisia tridentata*), which induced the accumulation of defense-related proteinase inhibitors in the leaves of tomato plants growing in the near vicinity (Farmer and Ryan, 1990). In the acceptor plants, the methyl-esters are hydrolyzed to yield either SA or JA, which in turn, induces the observed reactions. Considering these coherences, the experimental approach employed in this thesis indeed corresponds to a "natural situation"

In conclusion, there is an unimaginable high degree of variability of putative interactions and interferences between the JA and SA pathways. Accordingly, all changes in the kinetics of phytohormone biosynthesis and signaling in the course of the interactions of a plant might alter the biochemical defense responses and thus, the final outcome of the plant-herbivore or plant-pathogen interactions. These coherences may also explain the great variation and the contradictory results in the previous literature experiments, in which MeJA or SA are applied to indole alkaloid-containing plants, e.g., Aerts et al. (1996) and El-Sayed & Verpoorte (2004) reported a quite different and entirely contradicting effects when *C. roseus* seedlings have been treated with SA. However, we have to consider that these results are actually not equivalent, since the authors applied quite a different concentration of SA and, moreover, employed quite different times of incubation. Consequently, it is hardly surprising that the outcome of the defense response is completely different.

The stressed induced modification of indole alkaloids requires a novel classification of defense reactions

As outlined above, the known defense mechanisms are classified by two different types: in the case phytoalexins, the active substances are synthesized *de novo* from primary metabolites whereas the phytoanticipins are constitutively present in the plants (VanEtten *et al.*, 1994). The latter one either are active *per se*, i.e., the defense is directly due to the accumulated substance, or these substances are activated after cell death, i.e., by hydrolysis of the precursors (Matile, 1980; Tomas-Barberan and Gil, 2008; Iriti and Faoro, 2009). However, the results displayed in

the thesis, revealed that due to a stress-related elicitation, the MIAs are just slightly modified in vital, elicited cells. Yet, since they are not synthesized *de novo*, they do not represent classical phytoalexins and should be classified as phytoanticipins. However, their modifications do not occur postmortally but require a living, elicited cells. Hence, this type of defense compounds features characteristics of phytoalexins as well as of phytoanticipins, we have to introduce a new category, either within the class phytoanticipins by adding a sub-category of "substances modified in vital cells after elicitation" or within the class of phytoalexins as those being synthesized from complex precursors. Alternatively, a third main category apart from phytoanticipins or phytoalexins could be introduced. In this case, an appropriate denotation could be phytoelicitins.

Independently to the semantic problem, the modification of MIAs presented in this thesis vividly expound the large complexity and the high dynamic of plant defense reactions and the difficulty to incorporate and characterize them by simple classifications. In this context, it has to be noted that corresponding problems in the assessments or typecasting of a certain mode of defense represent a well-known issue, e.g., with respect to defense compounds: in grape wine, reveratrol is produced as typical phytoalexins in response to biotic elicitation (Chang *et al.*, 2011), but after successful defense of the pathogens, this stilbene is glucosylated and the corresponding glucoside, i.e., piceid, is stored in the vacuole and has to be considered as typical phytoanticipin with respect to a next infection. In the same manner, in tobacco plants, scopoletin is produced as phytoalexin in response to fungal or mosaic virus elicitors and accumulated as glycoconjugates without deleterious effects to the plant cell. Conjugation is also thought to provide a pool of inactive forms that can be rapidly transformed to active molecule via specific β-glucosidases in a secondary infection occurring in such tissues (Chong *et al.*, 1999; Costet *et al.*, 2002).

Furthermore, some compounds such as momilactone are known to be *de novo* synthesized in response to pathogen attack as a typical phytoalexin in leaves (Cartwright *et al.*, 1981), but are constitutively present as phytoanticipin in another part of the same plant (Kato *et al.*, 1973; Lee *et al.*, 1999). In the same sense, nicotine which is accumulated in the roots and leaves of *N. attenuata* as typical phytoanticipin, is – in addition - also *de novo* synthesized as phytoalexin in response to an attack by the larvae of *Manduca sexta* (Winz & Baldwin, 2001; Steppuhn *et al.*, 2004).

Conclusions and implication

Based on the insights mentioned above, it can be concluded:

The application of growth regulators or signal transducers involved in the signal transduction of stress responses could modulate the composition of secondary metabolites. Accordingly, elicitation could be a suitable, novel tool for further searches for pharmacologically active plant natural products. Such approach might open new doors to improve and facilitate phytochemical drug discovery.

In addition, also the application of enzyme inhibitors could be used as alternative approach to study but also to modify the biosynthetic pathway of certain secondary metabolite.

With respect to the biosynthesis of MIAs, the identification of the cytochrome P450 enzymes responsible for the stress induced conversion of indole alkaloids is an attractive topic for further studies.

5.3 Proposed biosynthetic pathway in the *V. minor*

As outlined above, the major indole alkaloids from *V. minor* belongs to three different classes, i.e., the eburnamine/vincamine-, the aspidospermine- and the akuammiline-group. Yet, all different structures are derived from strictosidine, resulting from the condensation of tryptamine and secologanin (Figure 5-10; Thamm *et al.*, 2016). The tremendous high variation in the indole alkaloid structures is due to modifications of the secologanin moiety, resulting in two different basic structures, i.e., the aspidosperma and the corynanthe/strychnos type (Figure 5-11). Moreover, the aspidosperma type skeleton could be rearranged, yielding in two different classes, representing the eburnamine/vincamine or the aspidospermine group.

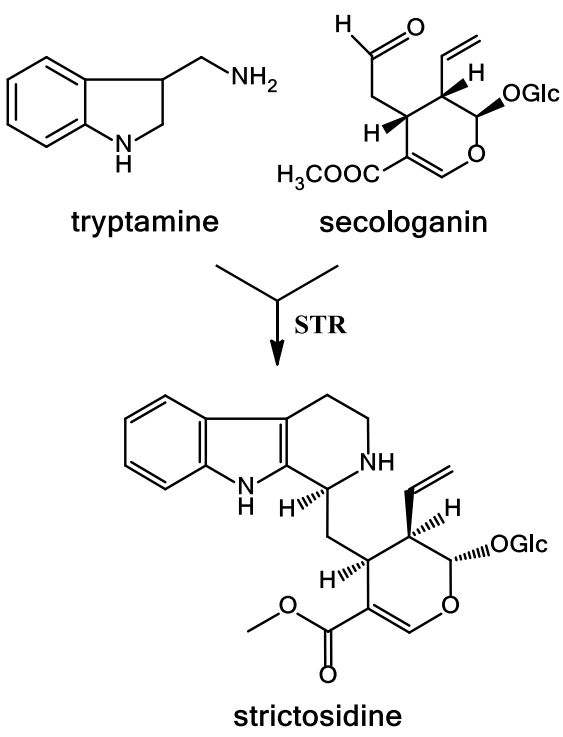


Figure 5- 10: Biosynthesis of strictosidine

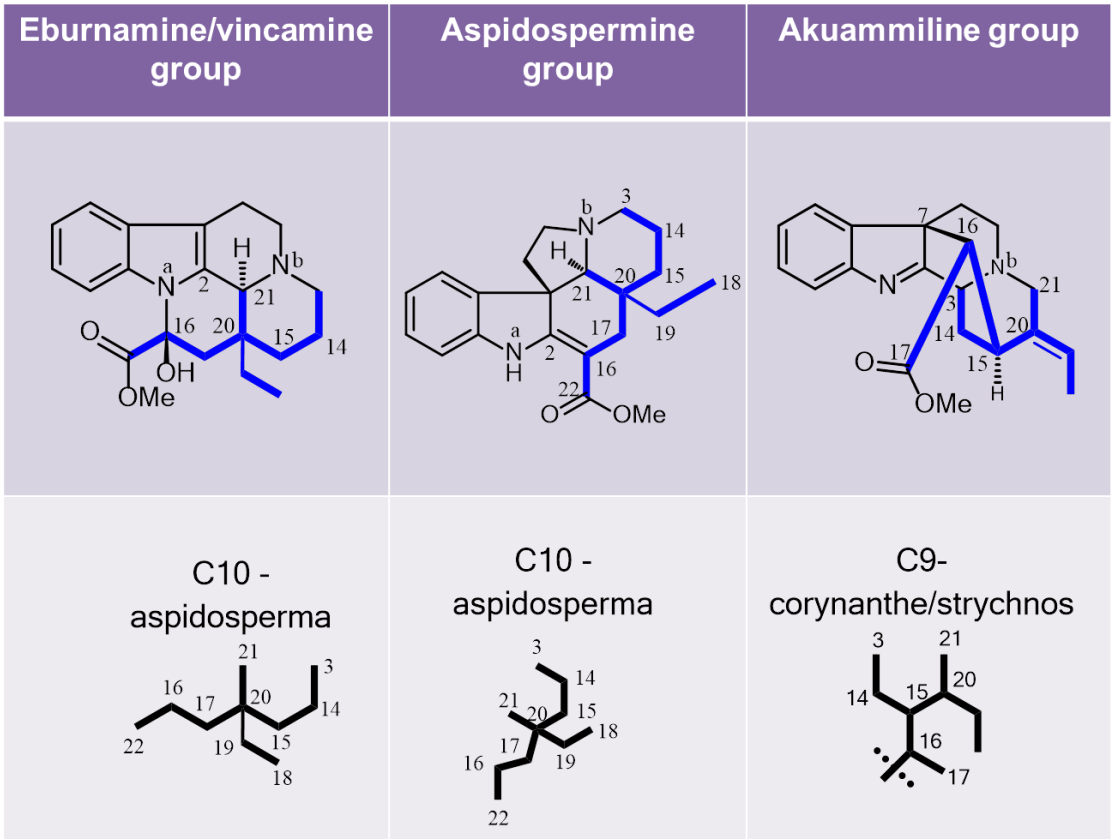


Figure 5- 11: The main groups of indole alkaloid in *V. minor*. The iridoid moiety, which results from various rearrangements of the secologanin skeleton, is displayed in bold.

In addition, also in the case of the aspidosperma alkaloids, one C-atom (C-22) of the rearranged secologanin moiety could be removed (Saxton, 1983a). Meanwhile, in many cases, the detailed routes of biosynthesis are elucidated; e.g., the aspidospermidine skeleton results from the attachment of C-21 to N_b and the β position of the tryptamine nucleus (the so-called β-condensation), followed by further cyclization between C-16 and C-2, leading to the aspidospermidine skeleton (Saxton, 1983a). In contrast, the eburnamine/vincamine skeleton is due to the attachment of C-21 to N_b and C-2 (α condensation), followed by cyclization between C-16 and N_a, leads to the (Saxton, 1983b).

With respect to the alkaloid biosynthesis in *V. minor* only limited information are available; nonetheless, it is known that also in this plant the MIAs are derived from the assembly of a secologanin and tryptamine (Thamm *et al.*, 2016). Scott (2002) proposed that geissoschizine is an early transformation product of strictosidine (Figure 5-12), which is thought to be an intermediate leading to all different MIAs and that strictamine results from an intramolecular oxidative coupling between the C7 and C16 atoms of geissoschizine followed by deformylation (Figure 5-12; Scott, 2002; Ren *et al.*, 2016). In addition, geissoschizine is postulated to be the precursor of stemmadenine that represents the putative intermediate leading to tabersonine (Qureshi and Scott, 1968b, 1968a) and vincadifformine. However, the detailed biosynthetic pathway is still not characterized (Qu *et al.*, 2018). Giddings *et al.* (2011) reported that in hairy root culture of *C. roseus* tabersonine is converted to vincadifformine via 6,7-reductase; nevertheless, also this step is uncharacterized at the genetic and enzymatic level.

Vincamine still exhibits a C10 aspidosperma unit; accordingly, it could be originated from vincadifformine by a series of reactions which involve the cleavage of bonds to both, the α and β positions of the indole (Wenkert and Wickberg 1965; Dewick 2009). This assumption was supported by feeding ^{14}C-labelled tabersonine to *V. minor* plants, which converted it to ^{14}C-vincamine (Kutney *et al.*, 1971). Kellner et al. (2015) suggested that the 2,3-epoxides tabersonine might be a common intermediate for the assembly of the eburnamine alkaloids such as vincamine by a non-enzymatic rearrangement. This assumption seems to be supported by approaches in the chemical semi-synthesis of vincamine from tabersonine or vincadifformine (Zsadon *et al.*, 1979; Saxton, 1983a; Szabó *et al.*, 1998; Li *et al.*, 2017). However, up to now, any enzyme or gene, respectively, involved in the biosynthetic pathway of vincamine in *V. minor* have not been identified yet.

In contrast, many enzymes and related genes are characterized in *C. roseus* (Giddings *et al.*, 2011; Qu *et al.*, 2015; Kellner *et al.*, 2015): in hairy root culture of *C. roseus*, vincadifformine could be transformed to minovincinine by the action of a cytochrome P450 enzymes, which specifically hydroxylates the 19-C of vincadifformine (Morgan and Shanks, 1999; Giddings *et al.*, 2011).

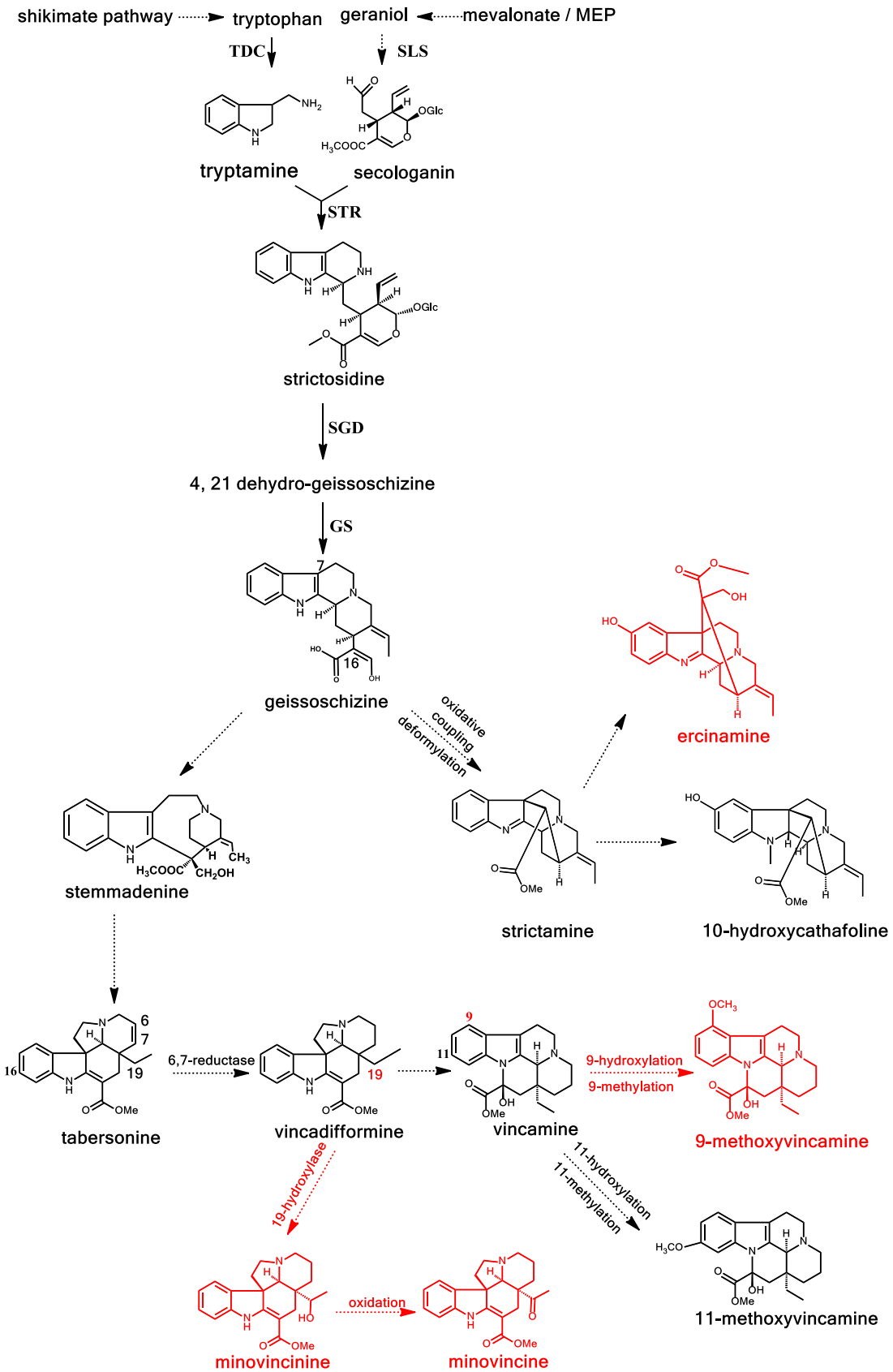


Figure 5- 12: Proposed biosynthetic pathway of indole alkaloids in *V. minor*. Based on the findings for *C. roseus* (Scott, 2002; Dewick, 2009; Giddings *et al.*, 2011; Qu *et al.*, 2018) and the deductions mentioned in the text, for the first time a corresponding pathways for the MIAs in *V. minor* under normal conditions (black) and under stress conditions (red) is presented. SLS: secologanin synthase; STR: strictosidine synthase; TDC: tryptophan decarboxylase; SGD: strictosidine β-glucosidase; GS: geissoschizine-synthase).

Moreover, Edge et al. (2018) reported that in *C. roseus*, in the course of vindoline biosynthesis, tabersonine is hydroxylated at 16-position (i.e. 6-position of indole ring) by a cytochrome P450 depending tabersonine-16 hydroxylase (Schröder *et al.*, 1999), followed by an *O*-methylation catalyzed by the 16-hydroxytabersonine-16-*O*-methyltransferase (Levac *et al.*, 2008). These results are in accordance with the finding outlined in the actual thesis, that in *V. minor* - under normal, unstressed conditions - the hydroxylation and methylation occur at 6-position indole ring, yielding in 11-methoxy-vincamine (see above Figure 5-12).

According to the published biosynthetic pathway for MIAs in *C. roseus* (Scott, 2002; Dewick, 2009; Giddings *et al.*, 2011; Qu *et al.*, 2018), a corresponding scheme for *V. minor* is proposed in Figure 5-12, in which also the results and insights from this thesis are incorporated. To highlight the stress-induced changes, the alkaloids, which are exclusively accumulated in response to the MeJA treatment, are displayed in red. In this sense, it becomes obvious that - in analogy to *C. roseus* - also in *V. minor* the 6-position of indole ring is hydroxylated and *O*-methylated under regular conditions. Accordingly, in *C. roseus*, tabersonine is converted to vindoline[8], whereas in *V. minor* vincamine is converted to11-methoxyvincamine[4]. In contrast, under stress conditions, vincamine is hydroxylated and *O*-methylated at the 4-position of indole ring, resulting in 9-methoxyvincamine. In analogy, vincadiffformine is hydroxylated at 19-position to form minovincine and subsequently oxidized to minovincine after MeJA treatment in *V. minor* leaves.

Based on the knowledge that resveratrol represents an effective inhibitor for cytochrome P450 enzymes - either due to its action as competitive substrate or as non-competitive inhibitor - (Piver *et al.*, 2001; Liu *et al.*, 2013; Martínez-Márquez *et al.*, 2016; El-Sherbeni and El-Kadi, 2016) this stilbene was employed in this thesis to modulate the alkaloids composition in stressed *V. minor*. As expected, the stressed induced changes in indole alkaloid composition were fully suppressed by the application of resveratrol to *V. minor* plants. In contrast, resveratrol applied via irrigated water had a minor effect on the composition of MIAs in the *V. minor* control plants. In principle, this could be due to three different effect:

1) there are no cytochrome P450 enzymes involved in the biosynthesis of the standard MIAs, present in accumulated plants
2) the concentration of resveratrol in the leaves are too low for an effective inhibition,
3) there are no changes detectable since the rate of alkaloid biosynthesis is too low.

[8] In vindoline, the 6-position of indole ring corresponds to the 16-position of standard numbering of the alkaloid, whereas in vincamine the 6-position of indole ring accounts for the 11-position of the alkaloid.

The first possibility, i.e., that there are no cytochrome P450 enzymes involved in the biosynthesis of MIAs in the *V. minor* control plants, seems to be very unlikely, since it is well known that in *C. roseus* many steps of the biosynthesis of the MIAs accumulated in the leaves and roots are catalyzed by cytochrome P450 enzymes, e.g., the hydroxylation at the 16- or 19-positions of tabersonine, (Furuya *et al.*, 1992; Shanks *et al.*, 1998; Morgan and Shanks, 1999; Giddings *et al.*, 2011). In contrast, the second possibility, i.e., that the concentration of resveratrol in the leaves are too low for an effective inhibition, could not be rejected out of hand; however we have to consider that the concentration of resveratrol applied in this work via the irrigation water reflects the same concentration, which was used by Tucker Serniak, (2016) to study allelopathic effects of resveratrol as well as that found in leachates of *Fallopia japonica*, which had been employed to horizontal natural product transfer (Fan *et al.*, 2009; Tucker Serniak, 2016; Radwan, personal communication). Accordingly, it could be assumed that resveratrol is taken up from the soil by the roots and accumulated in ample amounts in the leaves as reported earlier (Henry *et al.*, 2005; Fan *et al.*, 2009; Tucker Serniak, 2016). The most likely reason for the absence of a resveratrol effect on the composition of the MIAs in control plants concerns the low rate of biosynthesis within the plants during the treatment.

These coherences clearly point out, that in the future a putative effect of resveratrol on the composition of the MIAs in control plants and the elucidation of its biochemical background represent a promising and auspicious strategy. In addition, an alternative approach employing resveratrol is related to the horizontal natural product transfer: *Fallopia japonica* roots which contains large amounts of resveratrol and its glucoside (polydatin), respectively (Chen *et al.*, 2013) could be used to modulate the MIAs *V. minor*. The application of *F. japonica* roots, either as a mulching powder or via their aqueous extracts in the irrigation water seems to be an appropriate strategy to deliberately - but naturally - change to alkaloids composition in *V. minor* plants.

Conclusions and implication

Based on the insights mentioned above, it can be concluded:

The biosynthesis of MIAs in *V. minor* seems to be analogous to that of the alkaloids in *C. roseus*.

Stress induced by MeJA application alters to composition of MIAs via an induction of specific hydroxylation and *O*-methylation.

The key enzymes responsible for these changes and thought to be specific cytochrome P450 depending hydroxylases.

The identification and characterization of these enzymes should increase our understanding of the complex biochemical and ecological aspects of indole alkaloids; moreover, it will provide helpful tools to boost the discovery of new drugs or toxins.

5.4 New strategies in phytochemical drug discovery

Naturally occurring compounds play an essential role in drug discovery. From 1981 to 2014, 70% of all new approved therapeutic agents are traceable back to natural products (David *et al.*, 2015; Newman and Cragg, 2016). The exploration of natural biodiversity has led to the identification of a remarkable variety of chemical entities that reveal highly selective and specific biological activities and unique modes of action (Cragg and Newman, 2013). Bio-prospecting of natural sources is still of great interest for the discovery of new scaffolds; only 1% of tropical species have been investigated for their biological activities (Gurib-Fakim, 2006; Long *et al.*, 2014). Yet, routine and classical drug discovery programmes are based on plant natural products have mainly utilized secondary metabolites, which are often constitutive defense metabolites (Hostettmann *et al.*, 2005). Accordingly, the dynamic stress response of plants is usually not considered, although it is known that many phytoalexins also reveal strong bioactivities (Hammerschmidt, 1999). Consequently, in addition to the required analyses of so far non-investigated plants, a further approach to comprehensively using the entire potential of plants as valuable resources for pharmacological purposes has to be introduced: only when the ability of plants to respond to biotic and abiotic stress is adequately considered, the entire spectrum of natural products could be captured.

Dereplication-guided isolation of new indole alkaloids

To detect and identify bioactive compounds, efficient and innovative strategies are needed to significantly reduce the time-line of bioactive natural product discovery. All evidence suggests

that the most promising workflows for the chemical profiling of natural products should involve, at least in part, a dereplication procedure. Historically, the first definition of the term "dereplication" was given by Beutler et al. (1990) as "a process of quickly identifying known chemotypes". Accordingly, such approach also should be suitable for the high number of samples resulting from various elicitation procedures as outlined in detail below.

The goal of dereplication approaches was to evaluate the activity of a wide range of terrestrial and marine plant extracts and to rapidly identify compounds responsible for this activity without investing time in traditional bioassay-guided fractionation or full structure elucidation procedures (Hubert *et al.*, 2017). These methods are challenging to develop because they require high chromatographic resolution for detailed natural product profiling as well as high throughput for rapid quantification or fingerprinting analyses (Hubert *et al.*, 2017). In this respect, techniques such as LC-MS, mainly, have played key roles over the last three decades. The recent introduction of ultra-high pressure liquid chromatography (UHPLC) system coupled to ultra-high resolution time of-flight analysis in both the mass spectrometry (UHR-TOF-MS) and tandem MS (MS/MS) modes has represented an important breakthrough for the profiling of complex mixtures, such as natural extracts (Wolfender *et al.*, 2010). In comparison to conventional HPLC, UHPLC allows a remarkable decrease in analysis time as well as an increase in peak capacity, sensitivity and reproducibility (Eugster *et al.*, 2011). TOF-MS provides sensitive detection, high mass-resolution and high mass-accuracy for the rapid assignment of molecular formulae of natural products: a key piece of information for metabolite identification and dereplication. UHPLC-TOF-MS provides reproducible and highly informative datasets for MS-based metabolomics (Wolfender *et al.*, 2009). The most important aspect of this approach is the quick identification of known substances in order to concentrate the efforts on the discovery of new ones.

In this context, LC-MS with high accuracy and sensitivity has become a valuable method in dereplication guided isolation of novel MIAs (Aguiar *et al.*, 2010; Xu *et al.*, 2012; Y Liu *et al.*, 2015; J Liu *et al.*, 2015; Pan *et al.*, 2015; Akhgari *et al.*, 2015; Kumar *et al.*, 2016; J-G Zhang *et al.*, 2018). In this thesis, a simple, sensitive, reproducible UHPLC–ESI–MS/MS method was developed in positive ion mode for the dereplication of MIAs in *V. minor*. UHPLC profiling enabled the separation of a very high number of compounds and the detection of minor constituents. The high mass accuracy data generated by UHR-TOF-MS provides molecular formula information for all MIAs detected in stressed leaves sample of *V.* minor. The diagnostic fragmentation pathways were established with the help of MS/MS spectra of diagnostic fragment ions of isolated and NMR based identified compounds. Vincadifformine and

vincamine classes of MIAs showed two types of fragment ions, namely due to loss of substituents, which were attached with the terpene moiety and RDA fragment ions. The established diagnostic fragmentation pathways were applied for identification and characterization of other MIAs in the extract of stressed plants which were absent in the control. Extracted ion recording was used to detect the compounds with the same molecular ion, minor and co-eluting components.

About 20 alkaloids were tentatively detected and characterized in the extract of stressed leaves of *V. minor*. 9-hydroxyvincamine, 9-methoxyvincamine and 9-methoxyepivincamine were identified as novel alkaloids isolated and characterized for the first time. Moreover, other alkaloids had been described to occur in *V. minor* for the first time (e.g. ercinamine and 10-hydroxycathofoline).

The isomeric compounds of vincamine, methoxyvincamine, hydroxyvincamine, and vincaminine were successfully distinguished by MS/MS analysis. The present information showed the utility of the accurate mass measurements, which will also speed up dereplication of MIAs. It is important to mention that the ^{1}H and ^{13}C chemical shifts deviated significantly from literature data, probably due to the protonation of the basic nitrogen atoms by TFA or protonation of formic acid during HPLC fractionation. This illustrates that metabolite dereplication using carbon chemical shifts might be hampered in cases where the compounds act as weak acids or bases.

In conclusions, dereplication including the approaches outlined above seems to be an appropriate tool to analyze the complex profiles of extracts from elicitor-treated cells.

Stress induction for lead drug discovery

As outlined, it is well known that plants generate a large number of new, stress-induced natural products, which also might reveal high therapeutic value (Atkinson and Urwin, 2012; Wolfender and Queiroz, 2012). However, up to now, the various environmental impacts on the composition of active ingredients of medicinal plants had mostly been assessed negatively, mainly with respect of the abundant variations in the content of the active compounds. In this sense, the lack of reproducibility is considered as one of the major drawbacks in using plants in pharmaceutical discovery (Cordell, 2000). When considering the basic metabolic coherences in metabolism of plants exposed to various abiotic stress situations (Kleinwächter and Selmar, 2015), the observed differences in the concentration the various secondary plant metabolites in plants harvested at different times and locations become obvious (Poulev *et al.*, 2003; Atanasov

et al., 2015). Moreover, the biosynthesis of various defensive compounds, such as phytoalexins is strongly impacted by plant pathogens (Ahuja *et al.*, 2012).

Indeed, the consideration of all putative biotic and abiotic stress situations is quite impossible; however, due to the comprehensive knowledge of the underlying signal transduction mechanisms, the usage of certain elicitors, phytohormones and growth regulators represent effective tools for novel approaches.

Indeed, meanwhile, various elicitors already are frequently used to enhance the production of secondary metabolites. However, this strategy is strictly limited to increase the content of those compounds, which already have been present in the non-treated plants (Ferrari, 2011). Moreover, with respect to quality improvement of vegetables and fruits, e.g., by increasing the content of glucosinolates and flavanoid in broccoli (Ku and Juvik, 2013), or the enhancement of flavonoids in apple skin (Awad and Jager, 2002), the application of growth regulators is well established. However, up to now, corresponding approaches are not employed in drug discovery, e.g., to induce or enhance putative phytoalexins or phytoelicitins (see 5.2 page 160-161). This disregarding is peculiar, since the impact of elicitation on the bioactivity of many plant species is well established (Poulev *et al.*, 2003) as well as on the composition of the relevant compounds (Rijhwani and Shanks, 1998; Rodriguez *et al.*, 2003; Poulev *et al.*, 2003; Wojakowska *et al.*, 2013; Tang *et al.*, 2015; Sampaio *et al.*, 2016). Accordingly, the induction and the utilization of natural elicitation mechanisms should be introduced in forthcoming approaches in drug discovery.

We have studied the effect of different growth regulators or the inducers on the MIAs biosynthesis in *V. minor* to induce the production of new alkaloids through the activation of biosynthetic pathways encoded by genes that are silent under normal condition. Consequently, in this thesis, methyl jasmonic acid (MeJA), salicylic acid (SA), and hydrogen peroxide were used to induce characteristic stress responses and to impact the biosynthesis of indole alkaloids. As outlined, the application of MeJA and hydrogen peroxide resulted in massive changes in the pattern of indole alkaloids of *V. minor*, i.e., the appearance of novel, so far unknown indole alkaloids, whose bioactive properties are under study. Accordingly, the MeJA treatment of *Vinca* plants unequivocally proved that the elicitation by the application of growth regulators represents an appropriate and promising approach for identifying novel natural products, which might represent valuable pharmacological remedies.

Regulation of the biosynthesis of target bioactive compound

In addition to the application of elicitors, the treatment of the plant cell with enzyme inhibitor could be used as an additional approach to induce or modify the biosynthetic pathway of the

certain secondary metabolite by affecting the related metabolic fluxes. Such approach might be aimed to reduce the content of unfavorable compounds by inhibiting the enzymes involved in their biosynthesis, or, alternatively, to increase the concentration of the desired substances by inhibiting metabolic processes utilizing the same precursors.

In this sense, in *C. roseus* catharanthine formation was enhanced by minimizing the concurrent metabolic flux of its precursor, i.e., 4,21-dehydrogeissoschizine, to serpentine synthesis by using naproxen. This cyclooxygenase inhibitor strongly inhibited the biosynthesis of serpentine. As consequence, the metabolic flux to catharanthine biosynthesis was strongly enhanced, resulting in an elevated catharanthine concentration (Figure 5-13; Guo *et al.*, 2011). In another approach, the same authors demonstrated the high potential of such strategies by influencing the fluxes in alkaloid biosynthesis at three different sites (Guo *et al.*, 2013) to increase the vinblastine concentration.

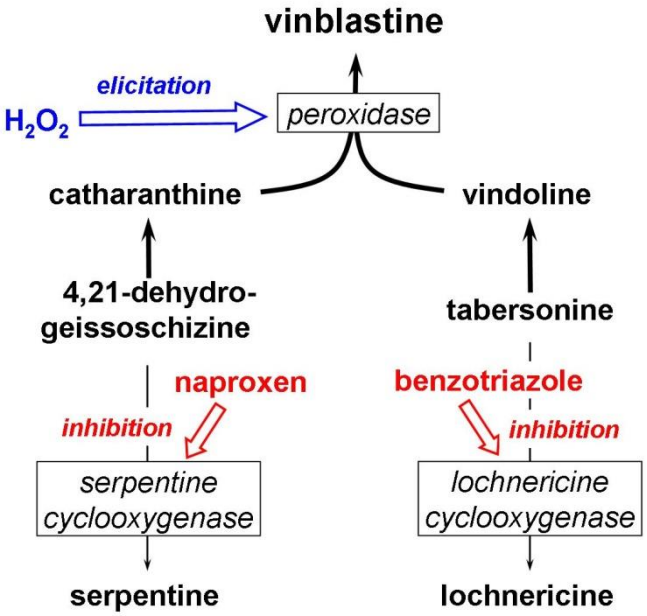


Figure 5- 13: Modulation of the vinblastine biosynthesis according to *Guo* et al. 2013.

In addition to the application of naproxen, which increased the metabolic flux to catheranthine, a second inhibitor, i.e., benzotriazole (Figure 5-13), which specifically inhibits the cyclooxygenase that generates lochnericine, was added to the cell cultures of *C. roseus*. In consequence, the metabolic flux from tabersonine into the lochnericine was strongly reduced, resulting in an elevated production of vindoline. Furthermore, into the medium, H_2O_2 was added, which is known to increase the generation of vinblastine by eliciting the peroxidase that is required for the condensation of vindoline and catharanthine at the final step of the vinblastine biosynthesis (Figure 5-13). In contrast, in hairy root cultures of *V. minor*, inhibitors (e.g. naproxen) had no effect, neither on total alkaloid nor on vincamine content. However, the combination of naproxen with H_2O_2 increases only the total alkaloid content (Verma *et al.*, 2014b). Therefore, the identifying of the enzymes involved in the alkaloid biosynthesis and ample understanding of the various signal transduction pathways in the modulation of secondary metabolites are vital for the further optimization of plant production systems.

According to Hasa et al. (2013), there is tremendous demand for novel indole alkaloid-based pharmaceuticals derived from *V. minor* extracts. As the vincamine present in the remedies from *Vinca minor* reveal a much higher oral bioavailability than pure vincamine, it is argued that certain, so far unknown, phyto-complexes might be involved in the bioavailability. Accordingly, there is a high demand for preparations from *V. minor*, which reveal strongly enhanced vincamine concentrations. Based on the coherences mentioned and the results outlined in this thesis, a corresponding auspicious approach could be based on the simultaneous application of growth regulators (MeJA or SA) and resveratrol as a potential inhibitor of certain cytochrome P450 enzymes. In consequence, due to the general elicitation, the overall content of alkaloids should be massively enhanced, but, due to the inhibition of hydroxylases responsible for the conversions outlined in Figure 4-18, no stress related derivatives of the basic MIAs will be formed and, accordingly, the vincamine concentration will increase. With respect to the potential activity of the related remedies, this approach might be even more advantageous, since resveratrol is known to be heath beneficial anyway, as it improves memory performance in association with improved glucose metabolism in the brain and might enhance the action of vincamine (Witte *et al.*, 2014; Hui *et al.*, 2018). Accordingly, the presence of both, high concentrations of vicamnine, and resveratrol, or its metabolites, in a standardized extract of *V. minor* could indeed be a promising approach to improve the bioactivity of *V. minor*.

Conclusions and implication

Based on the insights mentioned above, it can be concluded:

Dereplication-guided isolation of new indole alkaloids could promote the discovery of novel drugs in extracts from elicitor-treated cells.

The quantity as well as the composition of alkaloids accumulated in healthy and non-stressed plants could be altered massively by elicitation employing various growth regulators, e.g., MeJA, SA, H_2O_2. Corresponding approaches, which up to now only had been considered as a negative topic, are auspicious strategies to search for novel pharmacological active natural products.

The biosynthesis of certain bioactive compounds could be deliberately altered by the application of specific enzyme inhibitors, such as naproxen or resveratrol. Especially the combination of elicitation and the application of specific enzyme inhibitors seems to be a promising strategy for modulating the composition of biologically active natural products of a certain plant.

Chapter 6: Conclusion and future prospects

6.1 Conclusion

The scope of the current study was to investigate the impact of the application of growth regulators as a novel strategy to modify the composition of active components in medicinal plants. Accordingly, mature *Vinca minor* (Apocynaceae) plants of defined physiological status were used as a model system to investigate the effect of elicitation with methyl jasmonate (MeJA) and other growth regulators. The results presented in this work vividly demonstrate that such approach indeed leads to massive qualitative as well as quantitative variations in the content of bioactive compounds. In this context, MeJA and H_2O_2 treatment induces oxidative enzymes in *V. minor*, as suggested by the shifts in alkaloid distribution from vincamine to 9-methoxyvincamine and from vincadifformine first to minovincinine and finally to mino-vincine. The identification of the enzymes involved appears to be an attractive topic for further studies. In addition, the discovery of 9-methoxyvincamine as a novel natural product further implies that even well-known extensively studied plants such as *V. minor* might be an auspicious source for the generation of novel phytochemical drugs when grown under defined stress conditions. In consequence, this outcome highlights that the utilization of growth regulators or signal transducers, especially of MeJA, and the consideration of different developmental stages could be a promising tool for altering and diversifying the composition of active substances even in well investigated medicinal plants.

6.2 Future prospects

The current study represents an initial attempt to employ the growth regulators or signal transducers for the identification of novel pharmacologically active compounds and to optimization the corresponding phytochemical drugs. Moreover, it provides new tools for basic plant biology. In conclusion, some possible strategy can be identified as follows:

- The application of growth regulators might induce the biosynthesis, accumulation, and modulation of substances of therapeutic interest also in those plants, which- due to the absence of bioactivities – up to now, are only poorly studied. Accordingly, such approach might open new doors to improve and facilitate phytochemical drug discovery by providing an opportunity to explore minor constituents of poorly or extensively studied pants that can yield new bioactivities after chemical or biological manipulations.
- Dereplication-guided isolation from elicitor-treated plant extract could accelerate the efficient identification of new drug leads. In addition, developing a metabolomics

method for finding stress metabolites provides a good way to target the isolation of compounds of interest based on the MS characteristics of the stressed plant chemical profile.

- Evaluation of biological activities of the modulated plant extracts.

- The application of enzyme inhibitors could be used as an alternative approach to study and to modify the biosynthetic pathway of the certain secondary metabolite in the plant of interest.
- With respect to the biosynthesis of MIAs, the identification of the cytochrome P450 enzymes responsible for the stress-induced conversion of indole alkaloids is an attractive topic for further studies. As the identification and characterization of these enzymes should increase our understanding of the complex biochemical and ecological aspects of indole alkaloids; moreover, it will provide helpful tools to boost the discovery of new drugs or toxins.

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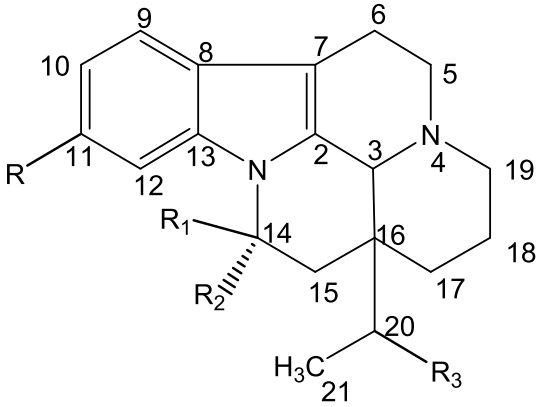
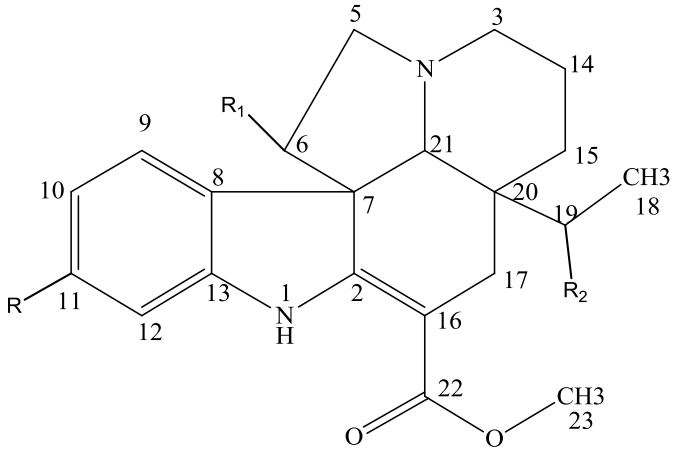
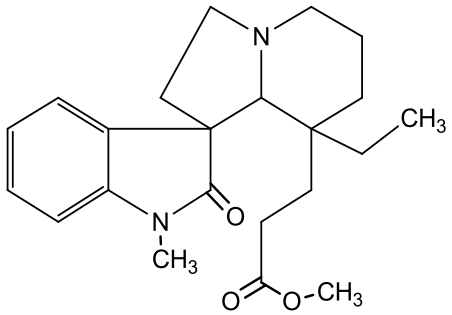
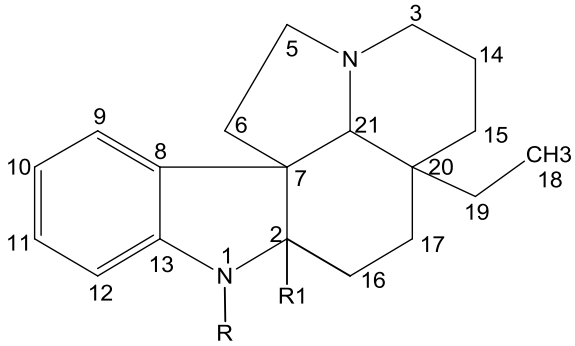
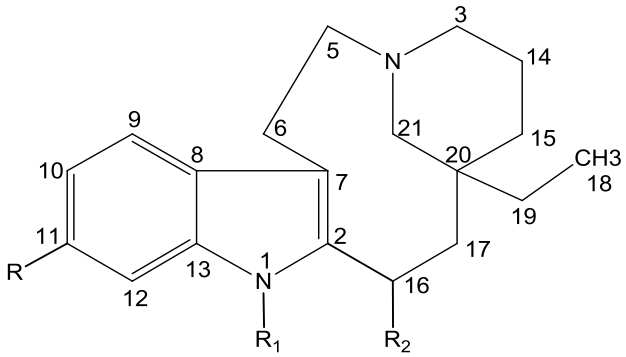
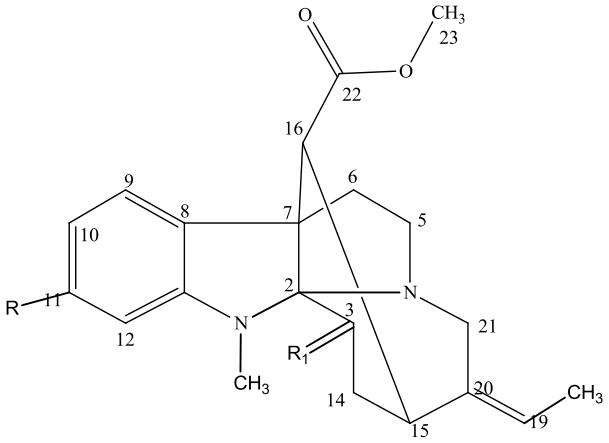
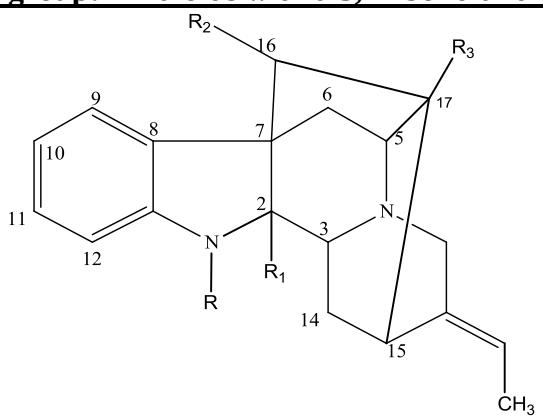
Appendix

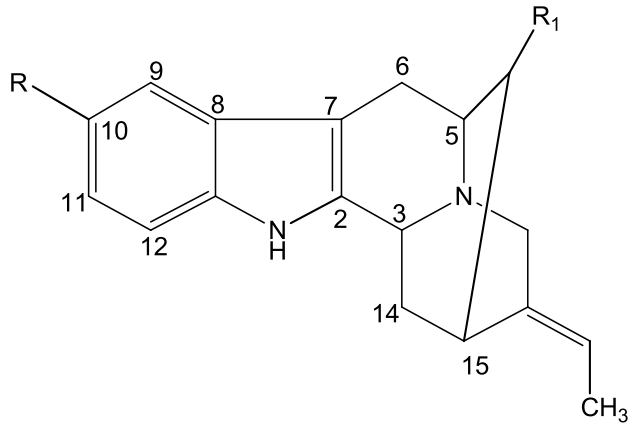
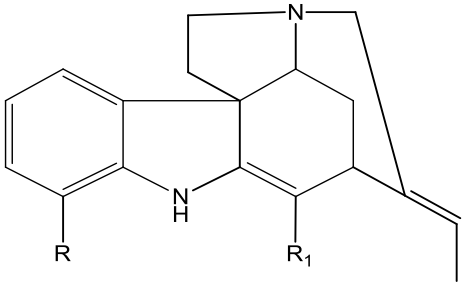
Table A- 1: Chemical structures of indole alkaloids isolated before from *V. minor.*

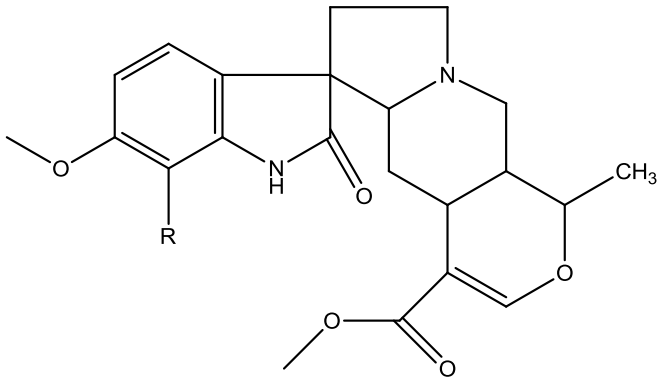
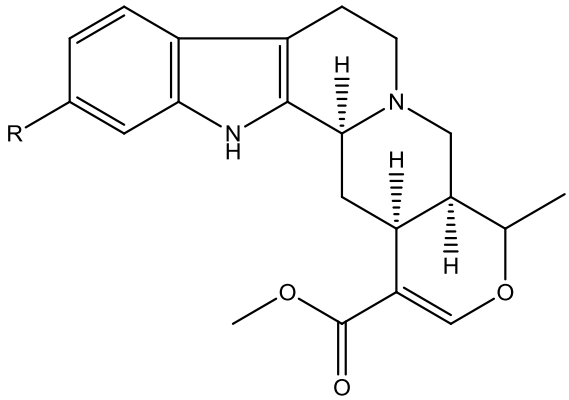
≠	Compound	Molecular formula/MW	R	References
A) <u>The Eburnamine-Vincamine Group</u> 				
1	Vincamine Syn. Minorine	$C_{21}H_{26}N_2O_3$ 354.448	R=R$_3$=H; R$_1$=OH, R$_2$=COOCH$_3$	(Proksa and Grossmann, 1991)
2	16-Epivincamine Syn. Isovincamine	$C_{21}H_{26}N_2O_3$ 354.448	R=R$_3$=H; R$_1$= COOCH$_3$; R$_2$=OH	(Taylor and Farn-Sworth, 1974)
3	(±)-Eburnamonine Syn. Vincamone, (-)-Eburnamonine, Vincanorine	$C_{19}H_{22}N_2O$ 294.396	R=R$_3$=H; R$_1$, R$_2$=O	(Mokry *et al.*, 1963; Proksa and Grossmann, 1991)
4	Vincaminol	$C_{20}H_{26}N_2O_2$ 326.43	R=H; R$_1$=OH R$_2$=CH$_2$OH,	(Kováčik and Kompiš, 1969)
5	Vincine Syn. 11-Methoxy-vincamine	$C_{22}H_{28}N_2O_4$ 384.47	R=OCH$_3$; R$_1$=OH; R$_2$=COOCH$_3$; R$_3$=H	(Trojánek *et al.*, 1961)
6	(-)-Eburnamine	$C_{19}H_{24}N_2O$ 296.41	R=R$_1$=R$_3$=H; R$_2$=OH	(Proksa and Grossmann, 1991)
7	(+)-Apovincamine	$C_{21}H_{24}N_2O_2$ 336.43	R=R$_3$=H; R$_2$=COOCH$_3$; $\Delta^{14,15}$	(Robakidze, 2003)
8	(+)-Eburnamenine	$C_{19}H_{22}N_2$ 278.39	R=R$_3$=H; R$_1$=H, $\Delta^{14,15}$	(Mokrý *et al.*, 1967)
9	(+)-Isoeburnamine	$C_{19}H_{24}N_2O$ 296.41	R=R$_2$=R$_3$=H; R$_1$=OH	(Mokrý *et al.*, 1967)
10	11-Methoxy-eburnamonine	$C_{20}H_{24}N_2O_2$ 324.422	R=OCH$_3$; R$_1$, R$_2$=O, R3=H	(Doepke *et al.*, 1968)
11	11, 12-Methoxy-eburnamonine	$C_{21}H_{26}N_2O_3$ 354.448	R=OCH$_3$; R$_1$,R$_2$=O, R$_3$=H	(Taylor and Farn-Sworth, 1974)

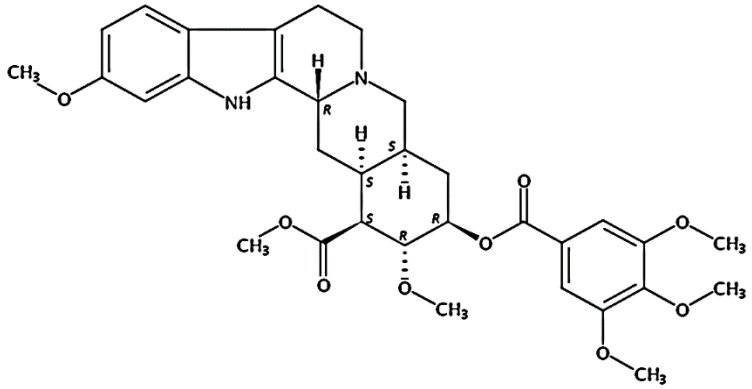
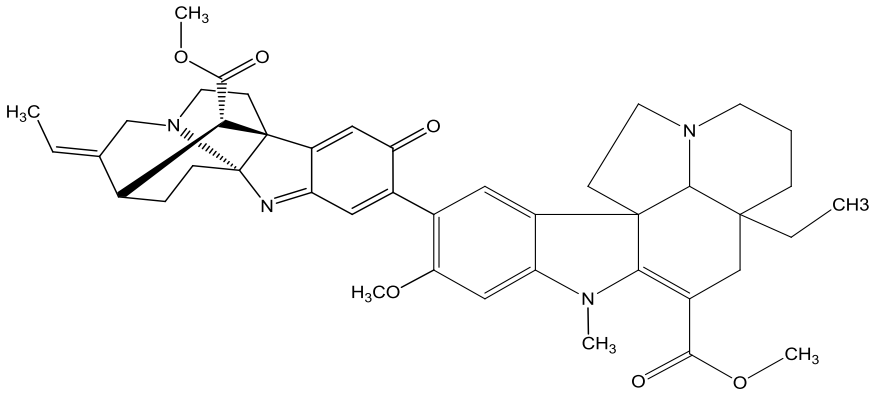
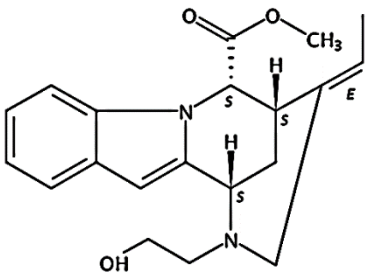
≠	Compound	Molecular formula/MW	R	References
12	Vincaminine Syn. 19-Oxo-vincamine, Vincareine	$C_{21}H_{24}N_2O_4$ 368.43	R= H; R_1=OH; R_2=COOCH; R_3= O	(Cekan *et al.*, 1962)
13	Vincinine Syn. 19-Oxo-11-methoxy-vincamine	$C_{22}H_{26}N_2O_5$ 398.45	R=OCH_3; R_1=OH; R_2=COOCH; R_3= O	(Cekan *et al.*, 1962)
14	Hydroxyvincamine	$C_{21}H_{26}N_2O_4$ 370.447	R= H; R_1= R_3=OH; R_2=$COOCH_3$	(Taylor and Farn-Sworth, 1974)
B) <u>Aspidospermine group</u> 				
15	(-)-Vincadifformine (±)-Vincadifformine	$C_{21}H_{26}N_2O_2$ 338.44	R=R_1=R_2=H	(Plat *et al.*, 1962; Proksa and Grossmann, 1991)
16	(-)-Methoxy-vincadifformine	$C_{22}H_{28}N_2O_3$ 368.47	R=OCH_3; R_1=R_2=H	(Doepke and Meisel, 1968)
17	(-)-Minovincinine	$C_{21}H_{26}N_2O_3$ 354.44	R=R_1=H; R_2=OH	(Plat *et al.*, 1962)
18	Minovincine	$C_{21}H_{24}N_2O_3$ 352.44	R=R_1=H; R_2=O	(Plat *et al.*, 1962)
19	Methoxy-minovincine	$C_{22}H_{26}N_2O_4$ 382.45	R=OCH_3; R_1=H; R_2=O	(Plat *et al.*, 1962)
20	(-)-Minovine Syn. N-methyl-vincadifformine	$C_{22}H_{28}N_2O_2$ 352.47	R=R_1= R_2=H N_1-CH_3	(Plat *et al.*, 1962)
21	5-Oxominovincine	$C_{21}H_{22}N_2O_4$ 366.41	R=H; R_1=R_2=O	(Taylor and Farn-Sworth, 1974)
22	(-)-Tabersonine	$C_{21}H_{24}N_2O_2$ 336.43	R=R_1=R_2=H; $\Delta^{14, 15}$	(Robakidze, 2003)

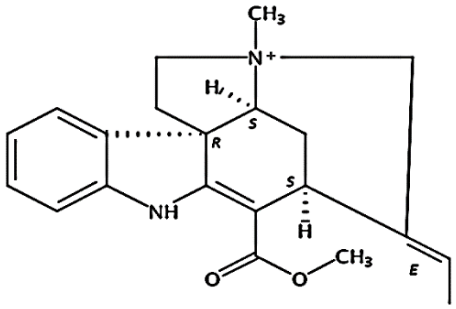
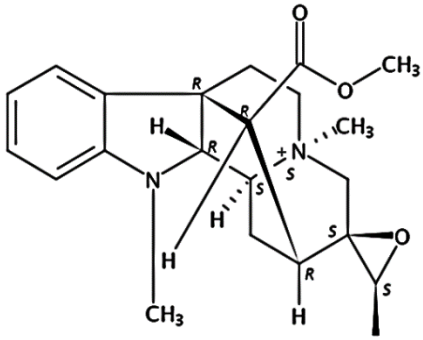
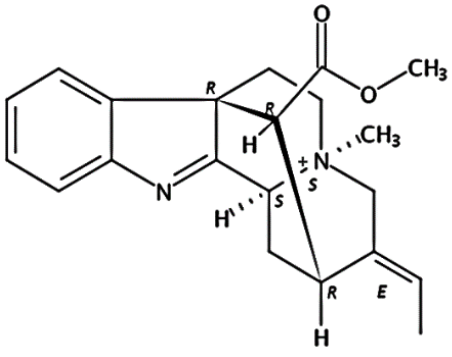
≠	Compound	Molecular formula/MW	R	References
23	(-)-Vincatine (oxindole alkaloid)	$C_{22}H_{30}N_2O_3$ 370.49 		(Doepke and Meisel, 1966; Doepke *et al.*, 1969)
				
24	(+)-*N*-Methyl-aspidosperimidine	$C_{20}H_{28}N_2$ 296.45	$R=CH_3$; $R_1=H$	(Mokrý *et al.*, 1967)
25	(+)-1,2-Dehydro-aspidospermidine Syn. Eburenine (root)	$C_{19}H_{24}N_2$ 280.41	$\Delta^{1,2}$	(Mokrý *et al.*, 1967)
				
26	(+)-Quebrachamine	$C_{19}H_{26}N_2$ 282.42	$R=R_1=R_2=H$	(Mokrý *et al.*, 1967)
27	(±)-*N*-Methyl-quebrachamine	$C_{20}H_{28}N_2$ 296.45	$R=R_2=H$, $R_1=CH_3$	(Mokry and Kompis, 1963)
28	(+)-Vincadine (leaves, root)	$C_{21}H_{28}N_2O_2$ 340.46	$R=R_1=H$, $R_2=COOCH_3$	(Mokry *et al.*, 1962)
29	(+)-Vincaminoreine (leaves, root)	$C_{22}H_{30}N_2O_2$ 354.49	$R=H$, $R_1=CH_3$, $R_2=COOCH_3$	(Trojanek *et al.*, 1960)

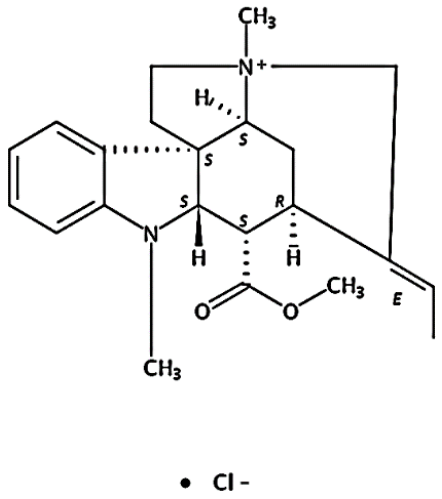
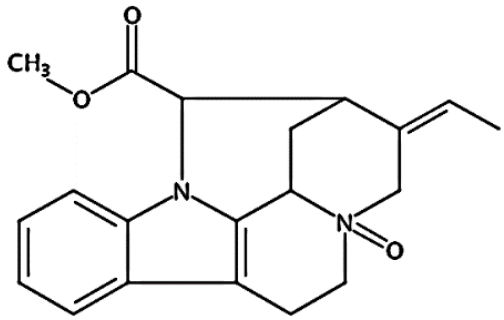
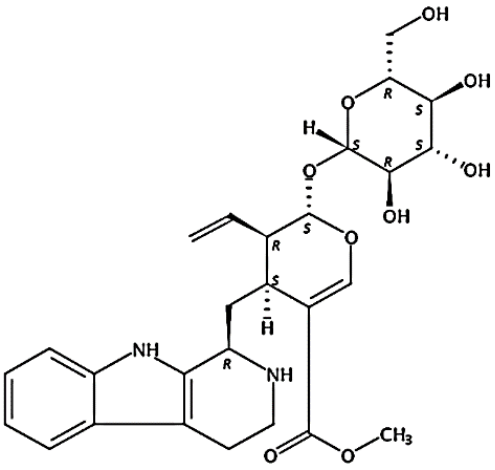
≠	Compound	Molecular formula/MW	R	References
30	(+)-Vincaminorine (leaves, root)	$C_{22}H_{30}N_2O_2$ 354.49	R=H, R_1=CH_3, R_2=$COOCH_3$	(Cekan *et al.*, 1960)
31	Vincaminoridine	$C_{23}H_{32}N_2O_3$ 384.51	R=OCH_3, R_1=CH_3, R_2=$COOCH_3$	(Mokrý and Kompiš, 1963)
C)	**<u>The akuammiline group: alkaloids with a 7,16 bond</u>**			
32	Strictamine Syn. Vincamidine (leaves)	$C_{20}H_{22}N_2O_2$ 322.40	R=R_2=H; $\Delta^{1,2}$ (**indolenine**)	(Mokrý *et al.*, 1967)
33	Picrinine (leaves)	$C_{20}H_{22}N_2O_3$ 338.40	R=R_2=H; 2,5-epoxy	(Grossmann *et al.*, 1973)
34	Deacetylakuammiline	$C_{21}H_{24}N_2O_3$ 352.43	R=H; R_2=CH_2OH; $\Delta^{1,2}$ (**indolenine**)	(Savaskan *et al.*, 1972)
35	10-Methoxydeacetyl-akuammiline	$C_{22}H_{26}N_2O_4$ 382.45	R=OCH_3; R_2=CH_2OH; $\Delta^{1,2}$ (**indolenine**)	(Savaskan *et al.*, 1972)
D)	**<u>secoakuammilan group: alkaloids with a 7,16 bond</u>**			
				
36	Vincoridine	$C_{21}H_{24}N_2O_3$ 352.43	R=H, R_1=O	(Mokrý and Kompiš, 1963)
37	Vincovine (-)-Vincorine	$C_{22}H_{28}N_2O_3$ 368.47	R=OCH_3, R_1=H	(Mokry *et al.*, 1962)
E)	**<u>The quebrachidine group: Alkaloids with a 5,17 bond and a 7,16 bond</u>**			
				

≠	Compound	Molecular formula/MW	R	References
38	Vincamedine	$C_{24}H_{28}N_2O_4$ 408.49	R=CH_3, R_1= H, R_2=R_3=CH_3COO	(Trojanek *et al.*, 1960)
39	(-)-Vincamajine *N*-Methylquebrachidine *O*-Deacetylvincamedine	$C_{22}H_{26}N_2O_3$ 366.45	R=CH_3, R_1=H, R_2=OH, R_3=CH_3COO	(Tulyaganov and Nigmatullaev, 2000)
				
41	10-Methoxyvellosimine	$C_{20}H_{22}N_2O_2$ 322.40	R=OCH_3, R_1= CHO	(Taylor and Farn-Sworth, 1974)
42	Tombozine Syn. Vellosiminol	$C_{19}H_{22}N_2O$ 294.398	R=H, R_1= CH_2OH	(Raynaud *et al.*, 1970; Tulyaganov and Nigmatullaev, 2000)
				
43	Vincanidine	$C_{19}H_{20}N_2O_2$ 308.37	R=OH, R_2= CHO	(Tulyaganov and Nigmatullaev, 2000)
44	Vincanine	$C_{19}H_{20}N_2O$ 292.37	R=H, R_2=CHO	(Tulyaganov and Nigmatullaev, 2000)
45	(-)-Akuammicine	$C_{20}H_{22}N_2O_2$ 322.40	R=H, R_2= CH_3COO	(Tulyaganov and Nigmatullaev, 2000)

≠	Compound	Molecular formula/MW	R	References
				
46	Majdine	$C_{23}H_{28}N_2O_6$ 428.48	R=OCH_3	(Tulyaganov and Nigmatullaev, 2000)
47	Vinerine	$C_{22}H_{26}N_2O_5$ 398.45	R=H	(Tulyaganov and Nigmatullaev, 2000)
48	Vineridine	$C_{22}H_{26}N_2O_5$ 398.45	R=H	(Tulyaganov and Nigmatullaev, 2000)
				
49	Ervine	$C_{21}H_{24}N_2O_3$ 352.43	R=H	(Tulyaganov and Nigmatullaev, 2000)
50	Reserpinin	$C_{22}H_{26}N_2O_4$ 382.45	R=OCH_3	(Tulyaganov and Nigmatullaev, 2000)

≠	Compound	Molecular formula/MW	R	References
51	Reserpine (Root + leaves) $C_{33}H_{40}N_2O_9$ 608.68 			(Lyapunova and Borisyuk, 1961; Lyapunova, 1964)
52	(-)-Vincarubine (bisindole alkaloid) $C_{43}H_{50}N_4O_6$ 718.88 			(Proksa *et al.*, 1986)
53	(-)-Vinoxine (leaves, root)	$C_{20}H_{24}N_2O_3$ 340.42 		(Mokrý *et al.*, 1967)
54	Vinomine	$C_{19}H_{22}N_2O_3$ 326.394	Structure is not available in the literature	(Meisel and Doepke, 1971)
55	Vintsine	Structure is not available in the literature		(Chekan, 1964)
56	Vincesine	$C_{22}H_{32}N_2O_4$	Structure is not available in the literature	(Doepke and Meisel, 1966)

≠	Compound	Molecular formula/MW	R	References
57	4-Methyl-akuammicinium chloride **(quaternary alkaloid)**	$C_{21}H_{25}N_2O_2$ .Cl 372.89 		(Proksa *et al.*, 1989)
58	4-Methyl-raucubaininium chloride **(quaternary alkaloid)**	$C_{22}H_{29}N_2O_3$ .Cl 404.93 		(Proksa *et al.*, 1989)
59	4-Methyl-strictaminium chloride **(quaternary alkaloid)**	$C_{21}H_{25}N_2O_2$ .Cl 372.88 		(Proksa *et al.*, 1989)

≠	Compound	Molecular formula/MW	R	References
60	N^1-methyl-2β,16β-dihydroakuammicine N^4-methochloride **(quaternary alkaloid)**	$C_{22}H_{29}N_2O_2$ .Cl 388.93 		(Votický *et al.*, 1979)
61	Epipleiocarpamine-N^4-oxide	$C_{20}H_{22}N_2O_3$ 338.40 		(Votický *et al.*, 1979)
62	Vincoside	$C_{27}H_{34}N_2O_9$ 530.57 		(Nishibe *et al.*, 1996)

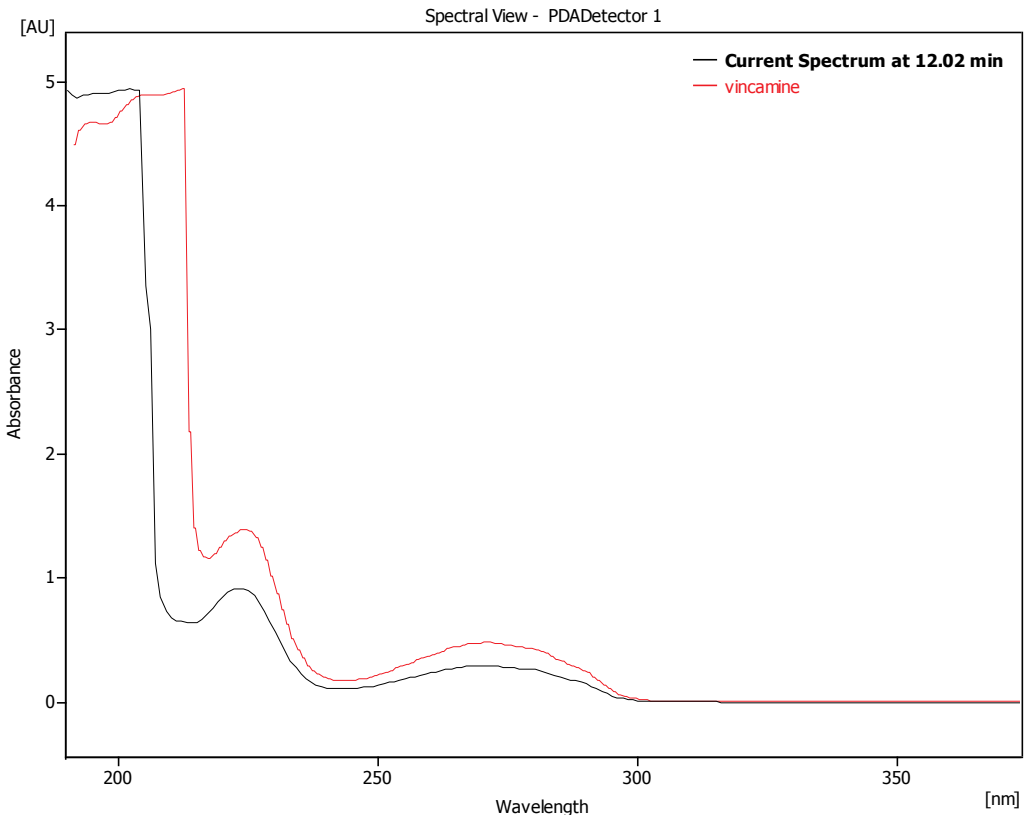
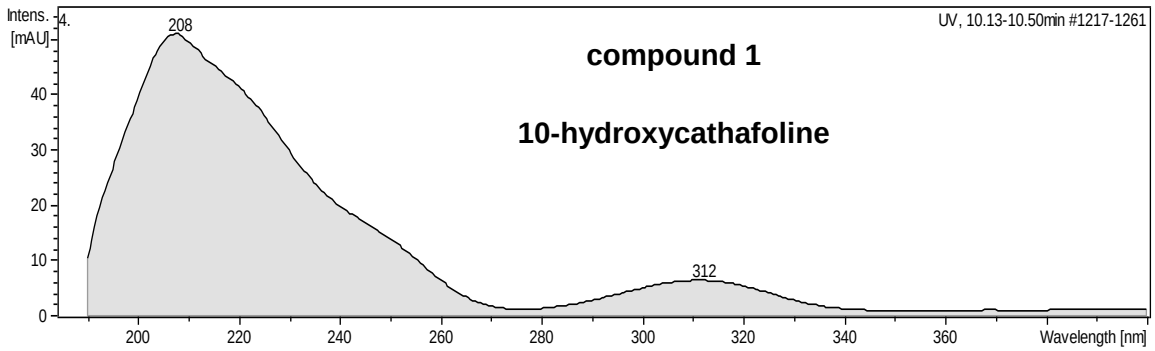
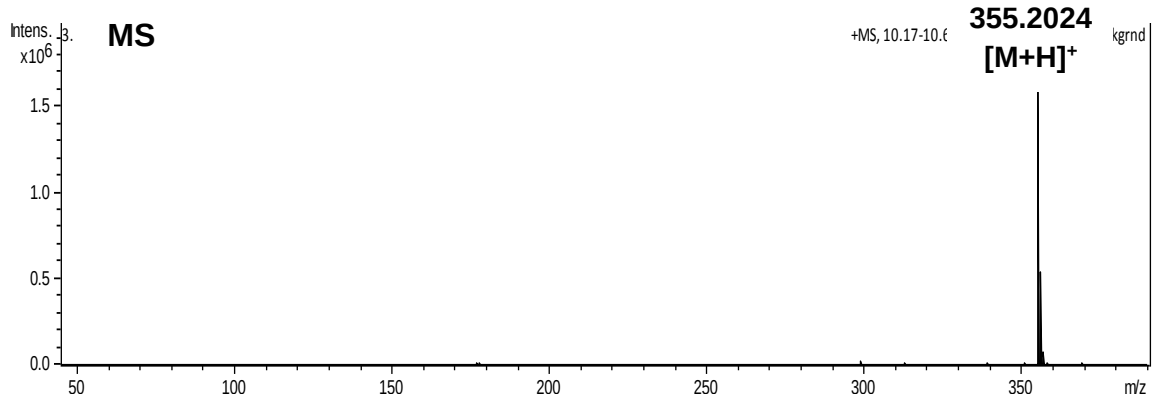
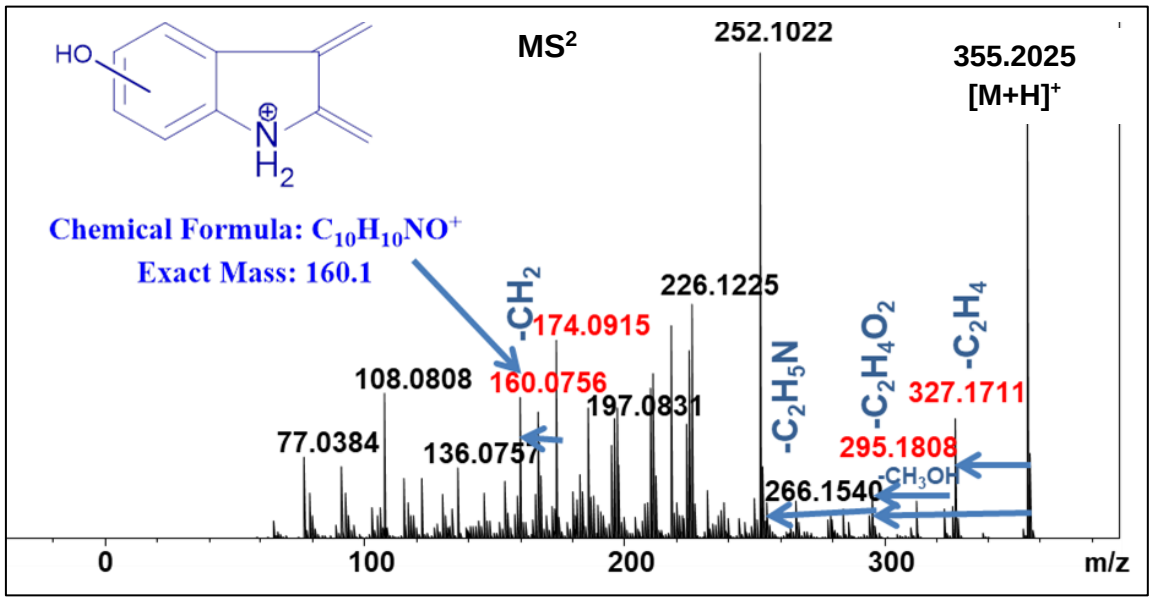


Figure A- 1: comparison between UV spectrum of vincamine standard and compound (2) in *V. minor* alkaloid extract







Spectrum Data

Meas. m/z	#	Sum Formula	Ion Formula	Adduct	m/z	err [ppm]	mSigma	# mSigma	Score
355.2022	1	$C_{21}H_{26}N_2O_3$	$C_{21}H_{27}N_2O_3$	M+H	355.2016	-1.6	5.9	1	100.00

Figure A- 2: UV, MS and MS-MS spectrum of 10-hydroxycathafoline (**1**).

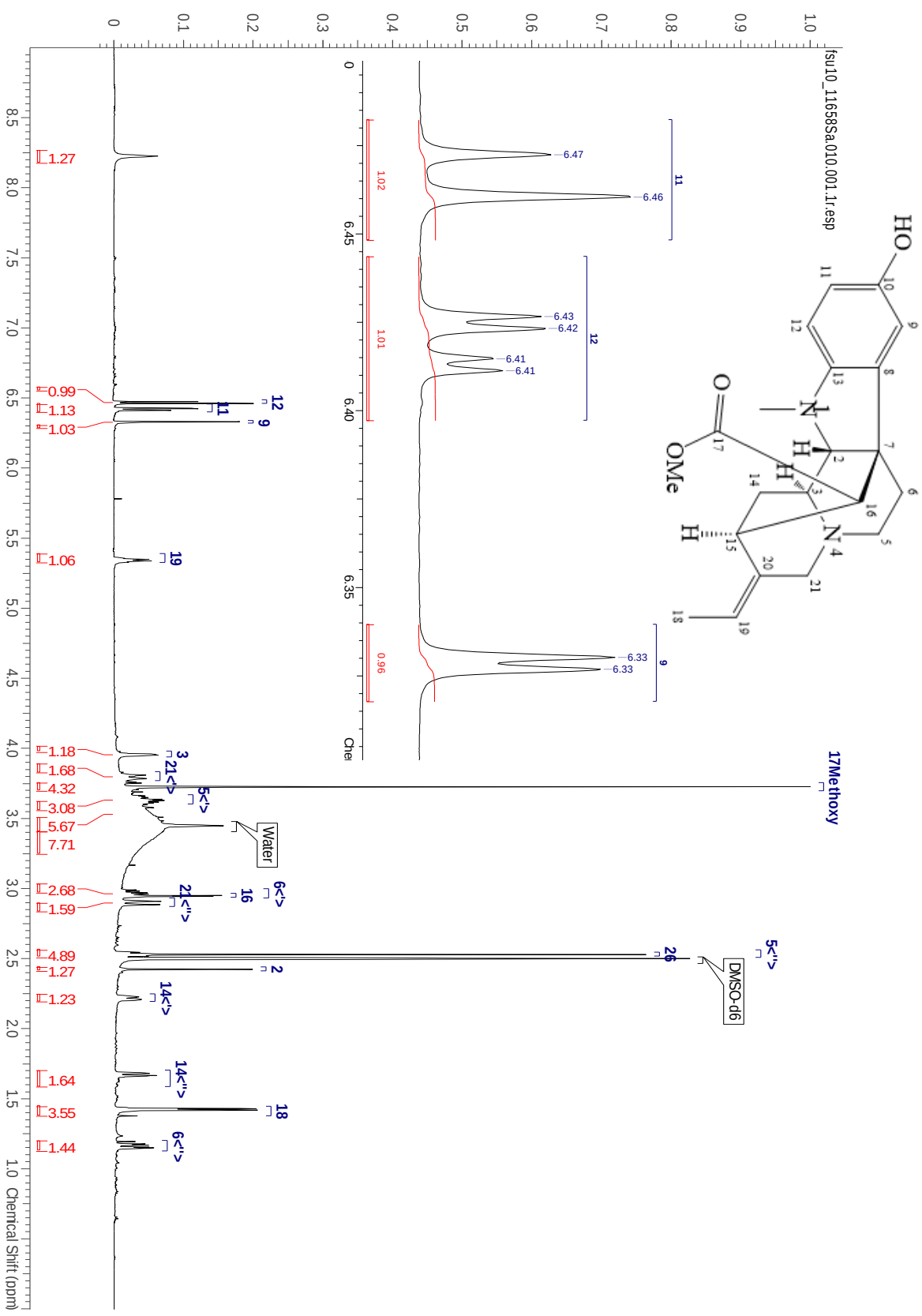


Figure A- 3: ^{1}H NMR spectrum (700 MHz, DMSO-d_6) of 10-hydroxycathofoline x formic acid (**1**).

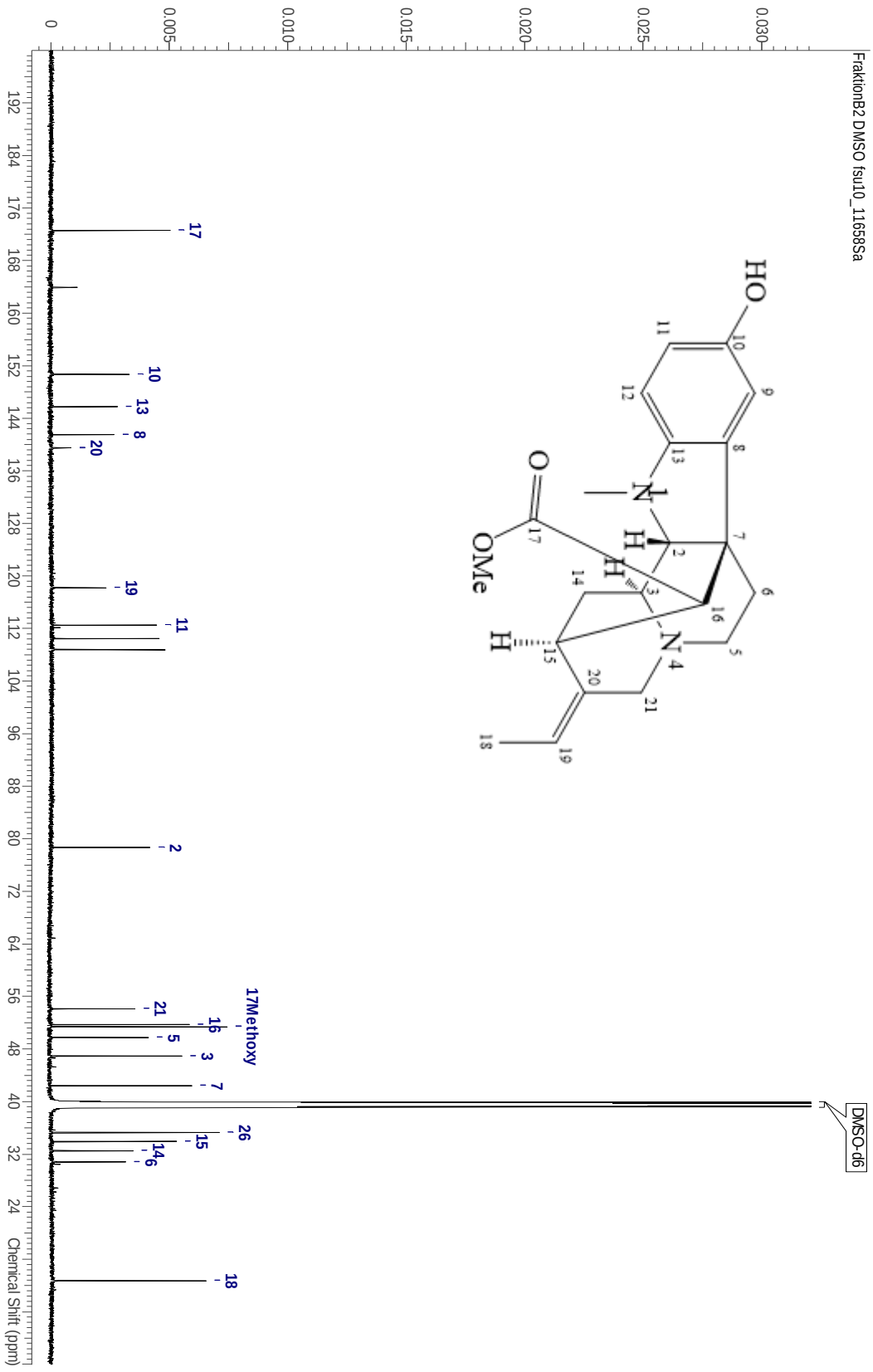


Figure A- 4: ^{13}C NMR spectrum (175 MHz, DMSO-d_6) of 10-hydroxycathofoline x formic acid (**1**).

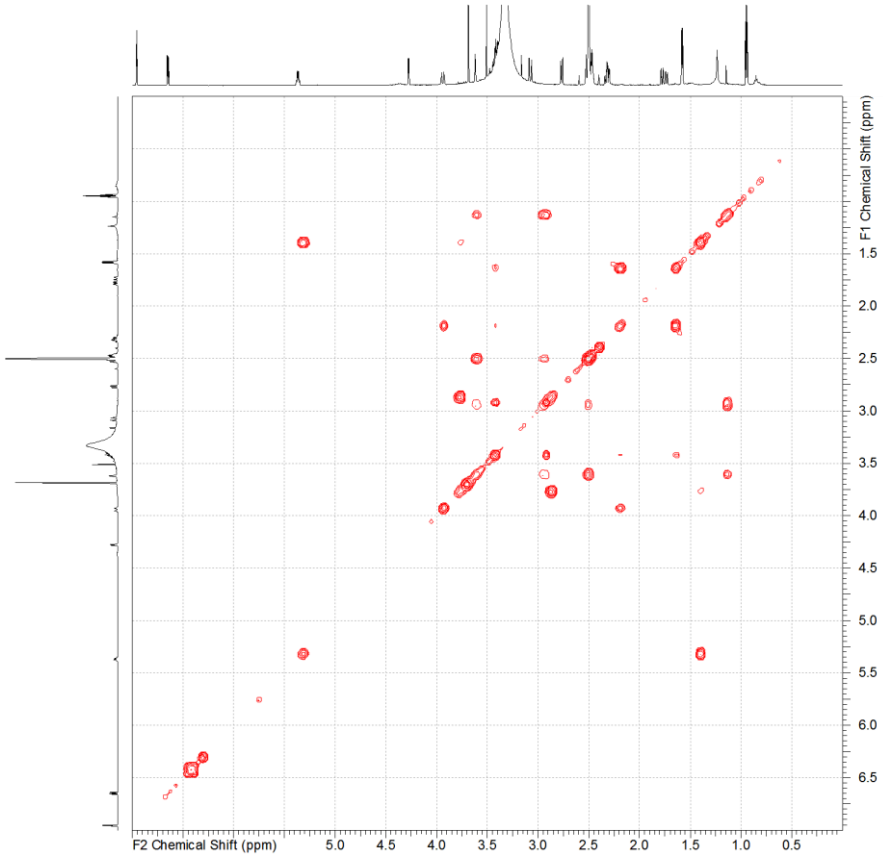


Figure A- 5: ^{1}H-^{1}H-COSY-NMR spectrum (700 MHz, DMSO-d_6) of 10-hydroxycathafoline **(1)** x formic acid.

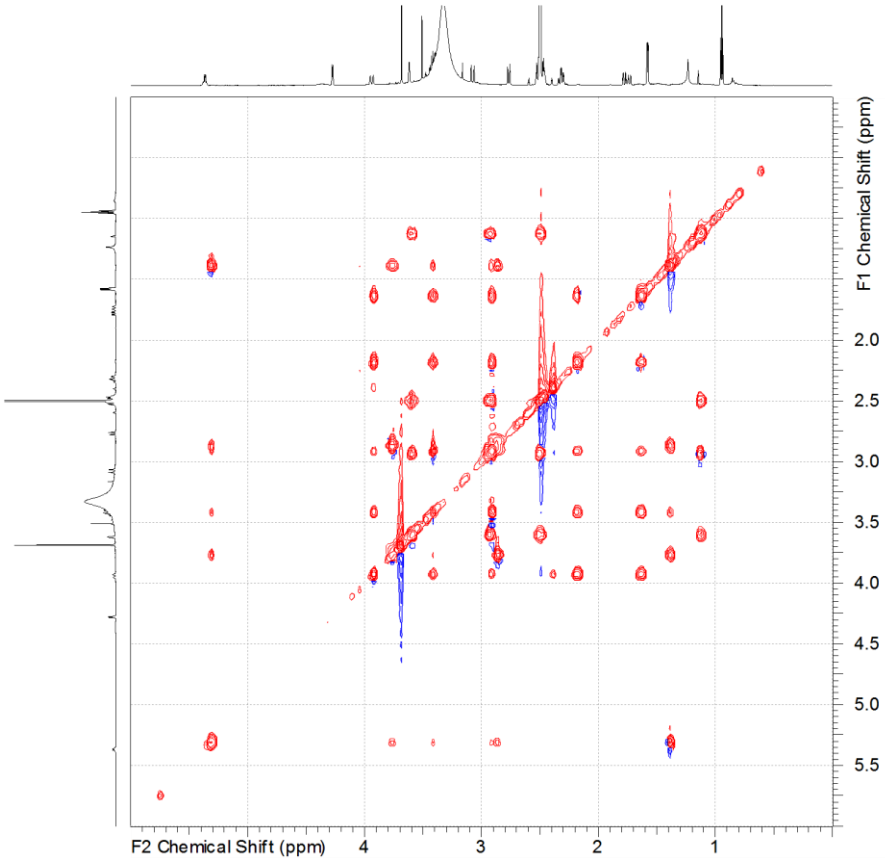


Figure A- 6: ^{1}H-^{1}H-TOCSY- NMR expansion spectrum (700 MHz, DMSO-d_6) of 10-hydroxycathafoline (**1**) x formic acid.

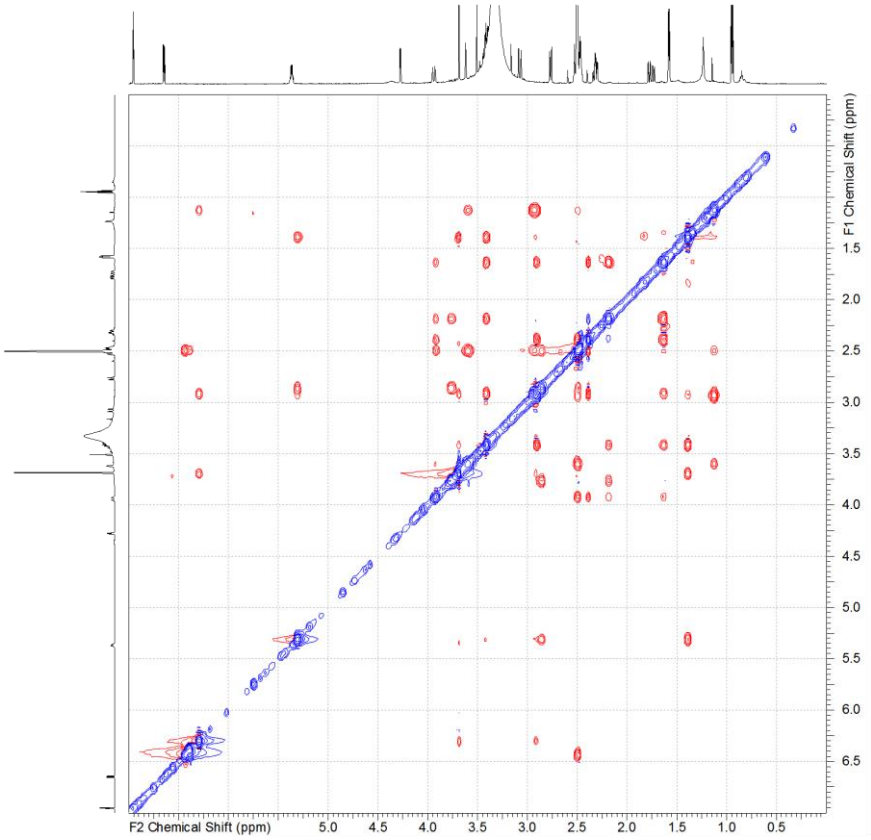


Figure A- 7: ^{1}H-^{1}H-ROESY-NMR spectrum (700 MHz, DMSO-d_6) of 10-hydroxycathafoline (**1**) x formic acid.

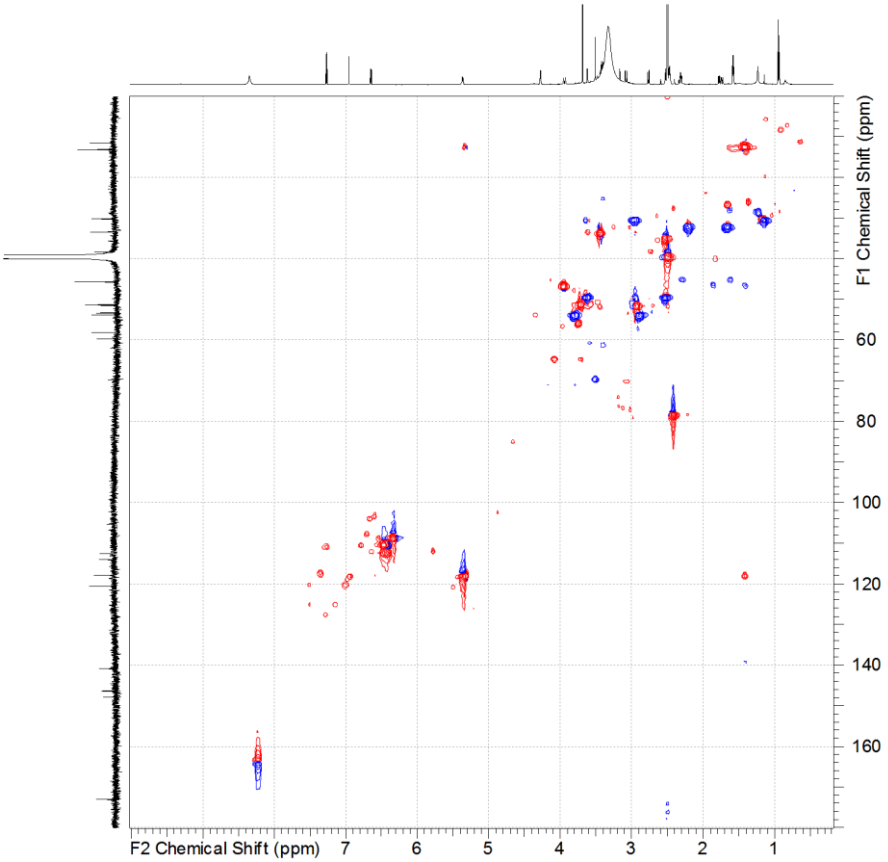


Figure A- 8: ^{1}H-^{13}C-HSQC-DEPT-NMR spectrum (700 MHz, DMSO-d_6) of 10-hydroxycathafoline (**1**) x formic acid.

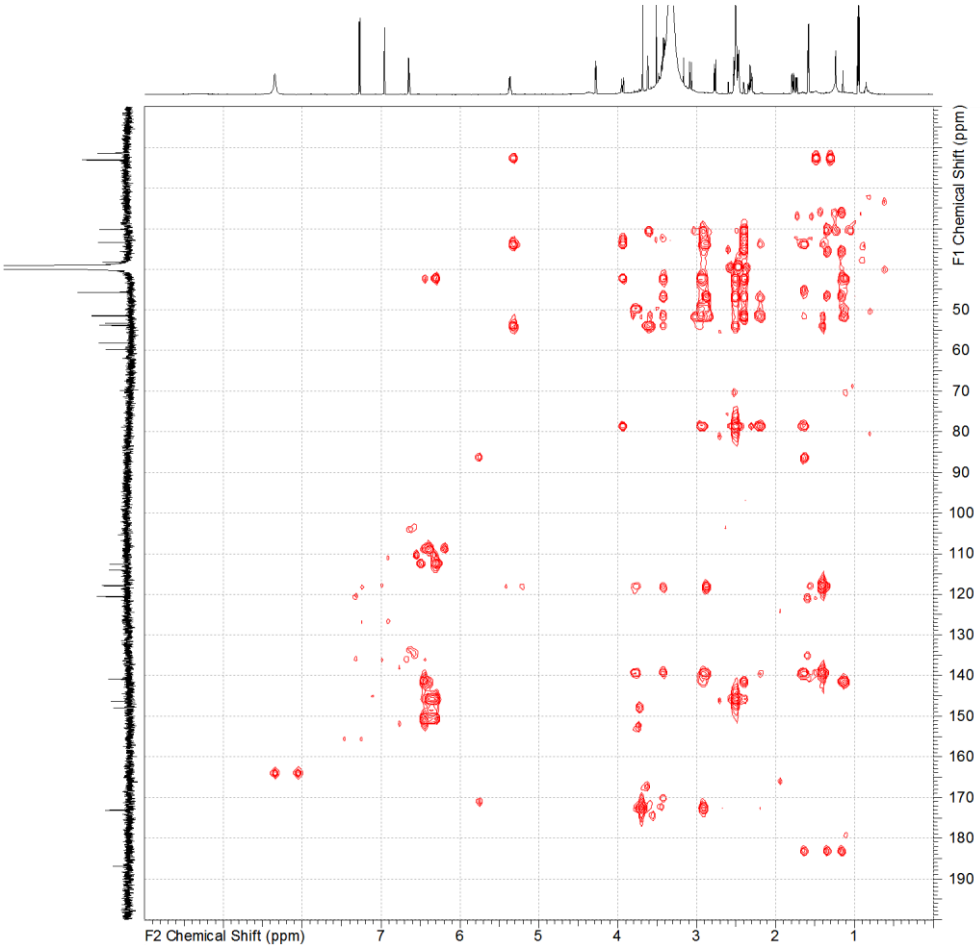
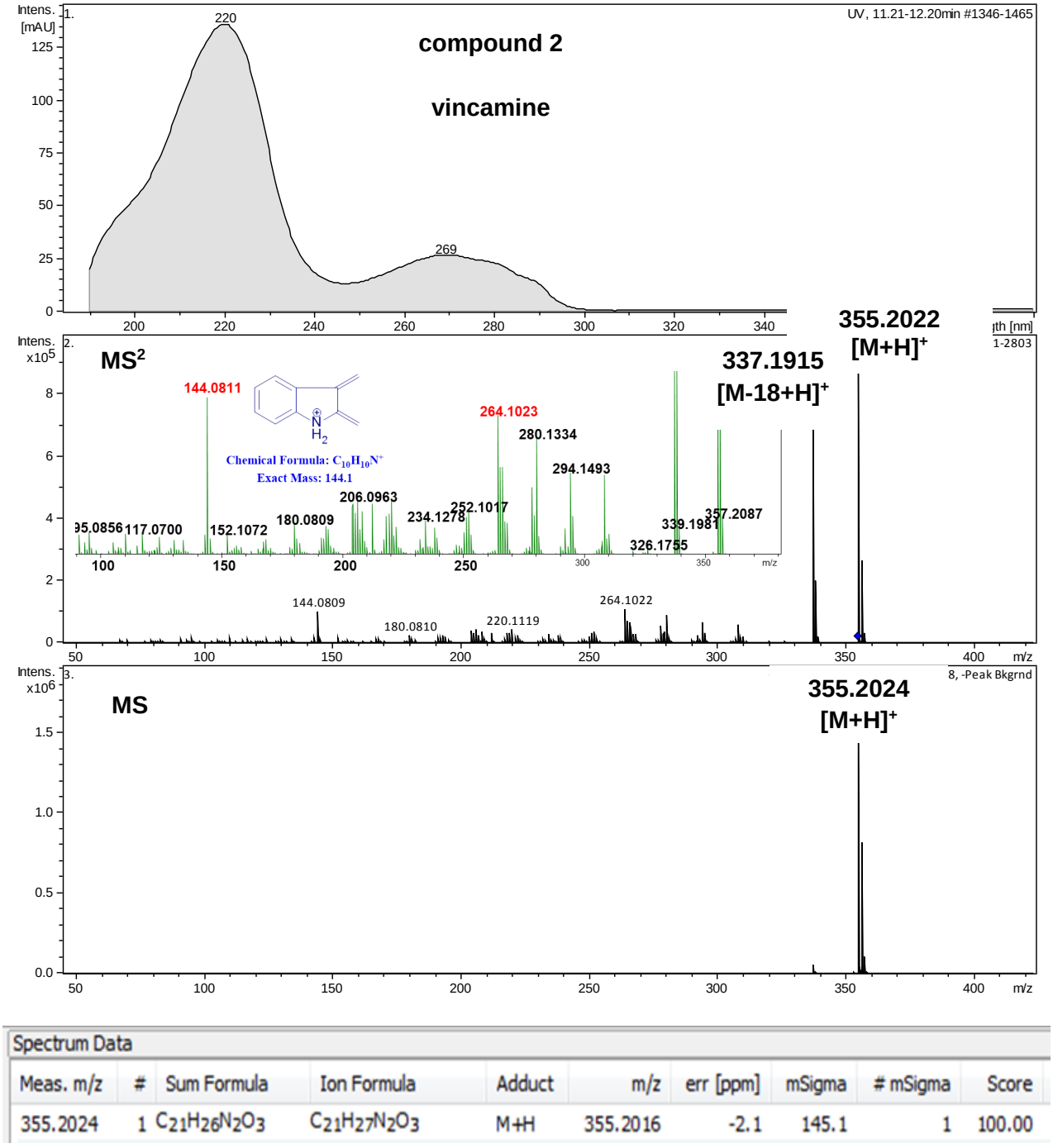


Figure A- 9: ^{1}H-^{13}C-HMBC NMR spectrum (700 MHz, DMSO-d_6) of 10-hydroxycathafoline (**1**) x formic acid.



Spectrum Data

Meas. m/z	#	Sum Formula	Ion Formula	Adduct	m/z	err [ppm]	mSigma	# mSigma	Score
355.2024	1	$C_{21}H_{26}N_2O_3$	$C_{21}H_{27}N_2O_3$	M+H	355.2016	-2.1	145.1	1	100.00

Figure A- 10: UV, MS and MS-MS spectrum of vincamine (**2**).

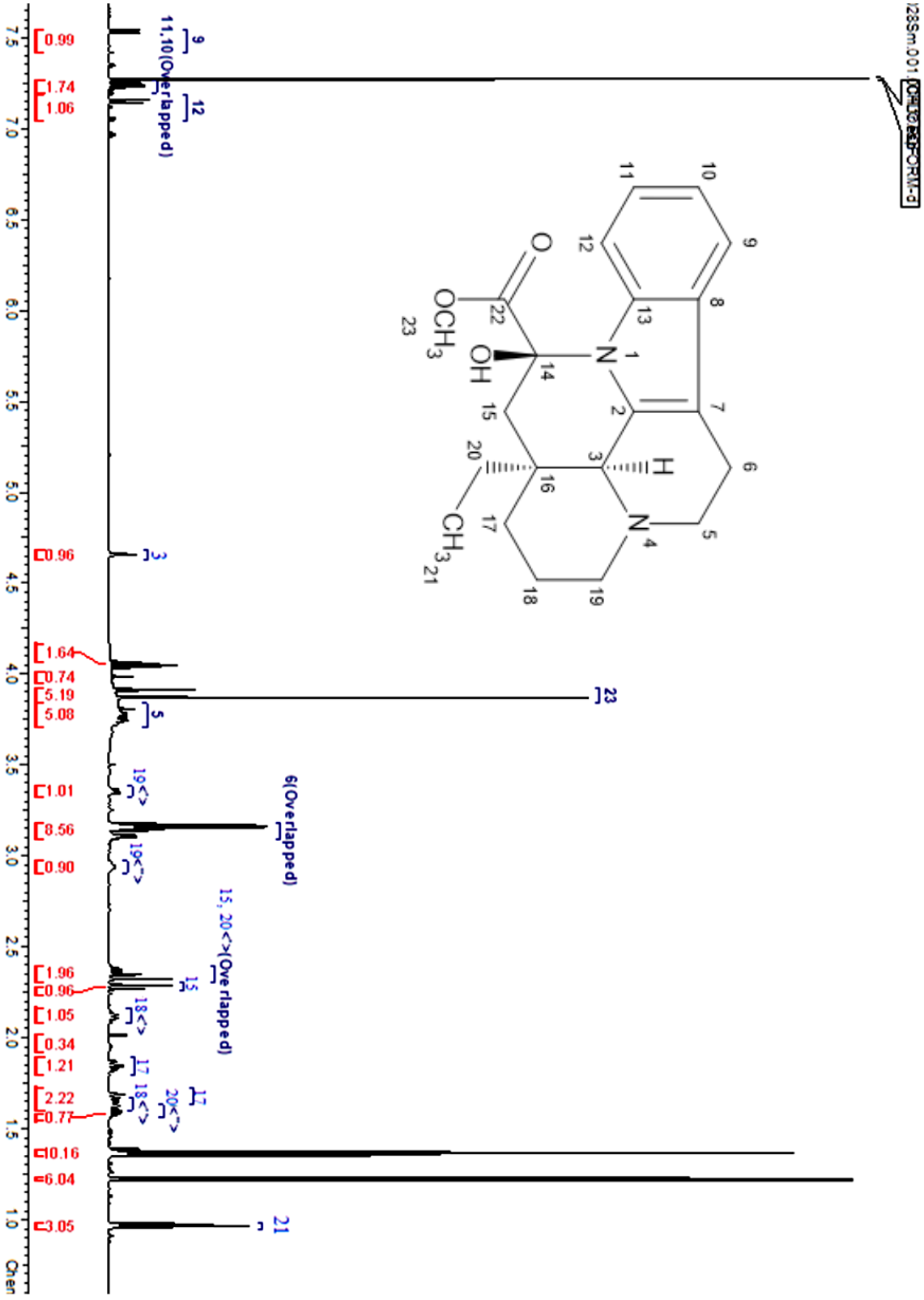


Figure A- 11: ^{1}H NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincamine (**2**) xTFA.

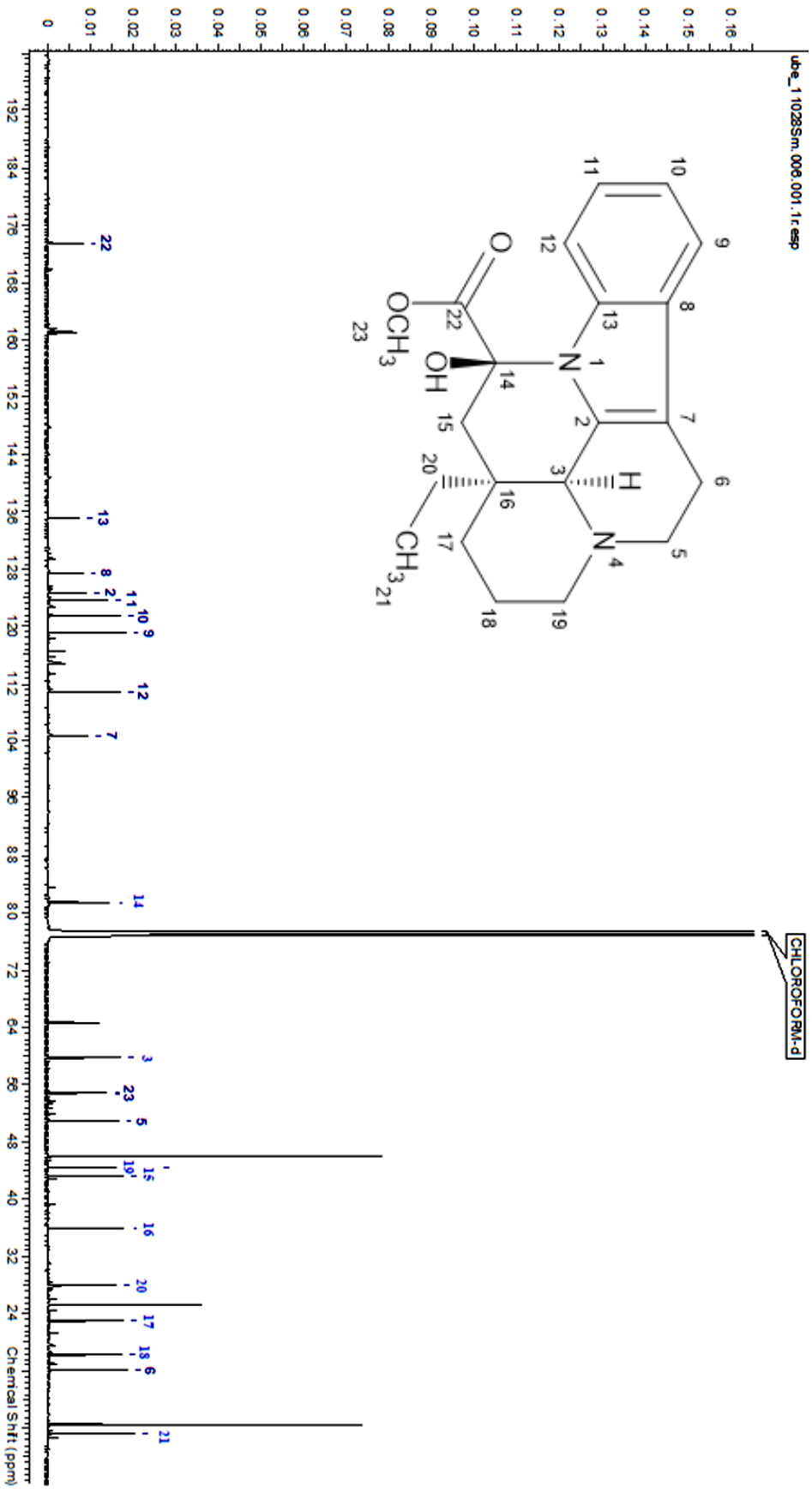


Figure A- 12: ^{13}C NMR spectrum (175 MHz, $CHCl_3$-*d*) of vincamine (**2**) xTFA.

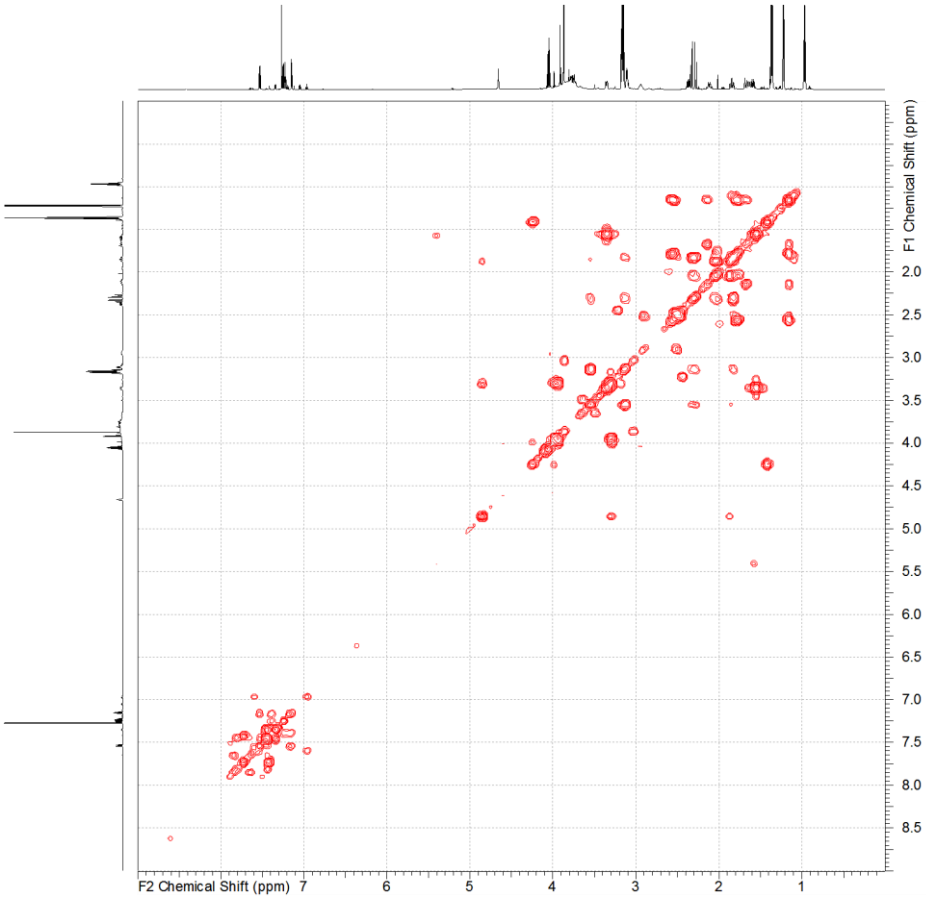


Figure A- 13: ^{1}H-^{1}H-COSY-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincamine (**2**) xTFA.

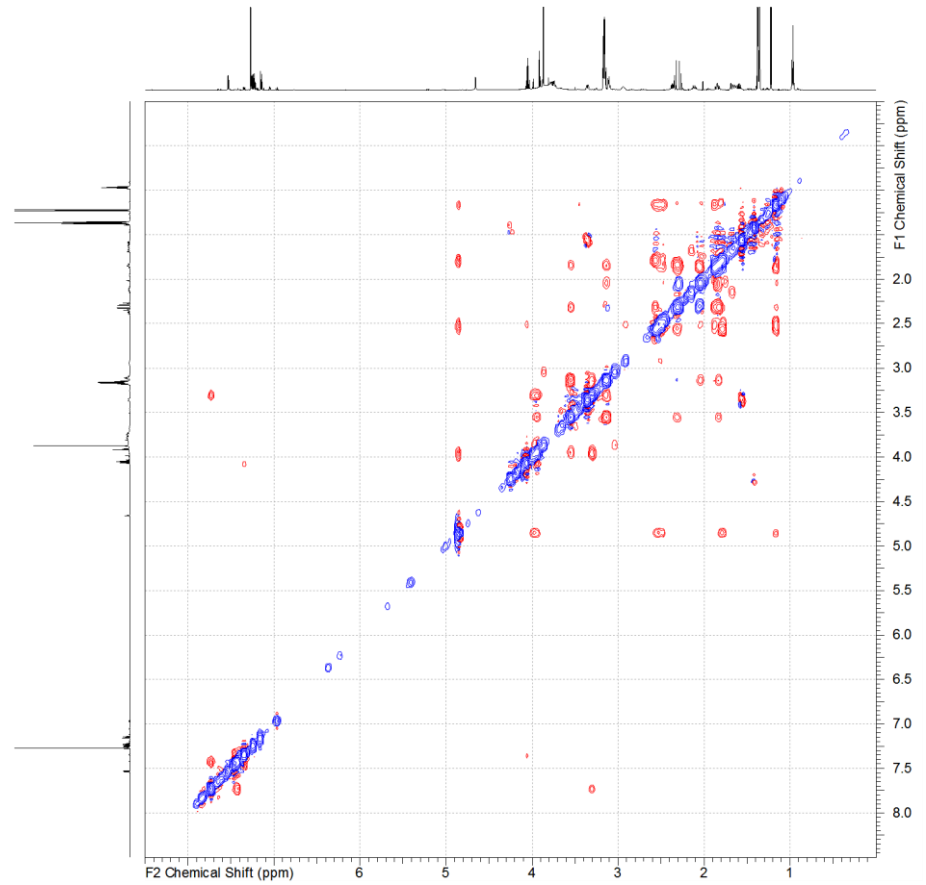


Figure A- 14: ^{1}H-^{1}H-ROESY-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincamine (**2**) xTFA.

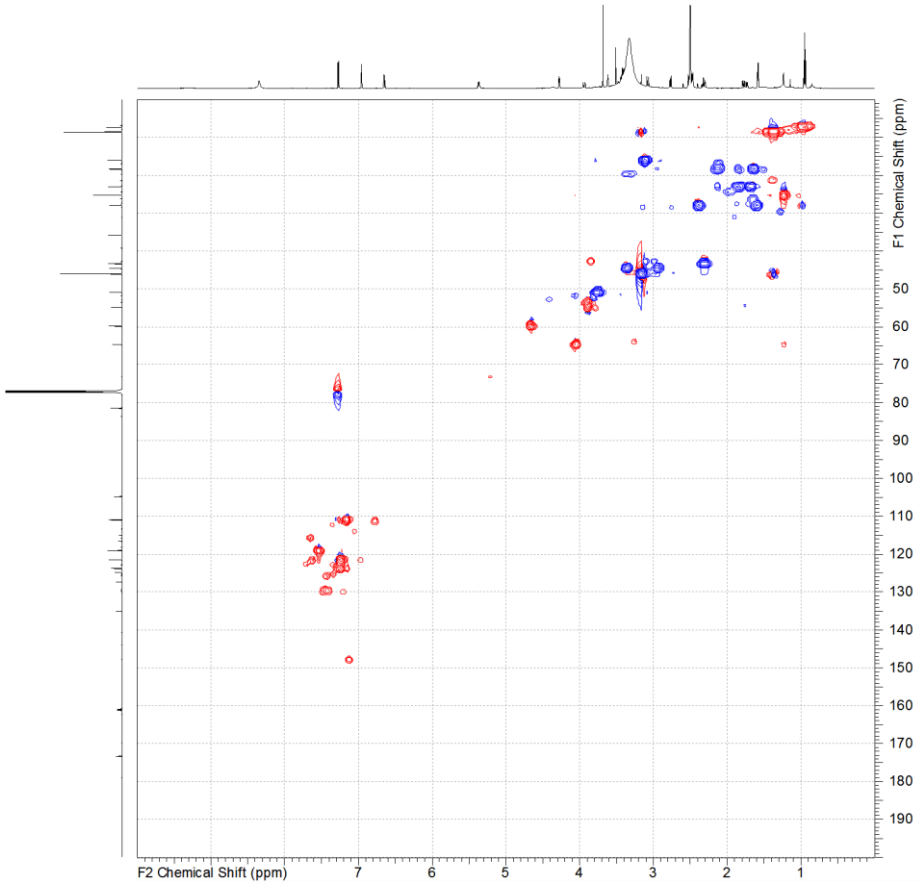


Figure A- 15: ^{1}H-^{13}C-HSQC-DEPT-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincamine (**2**) x TFA.

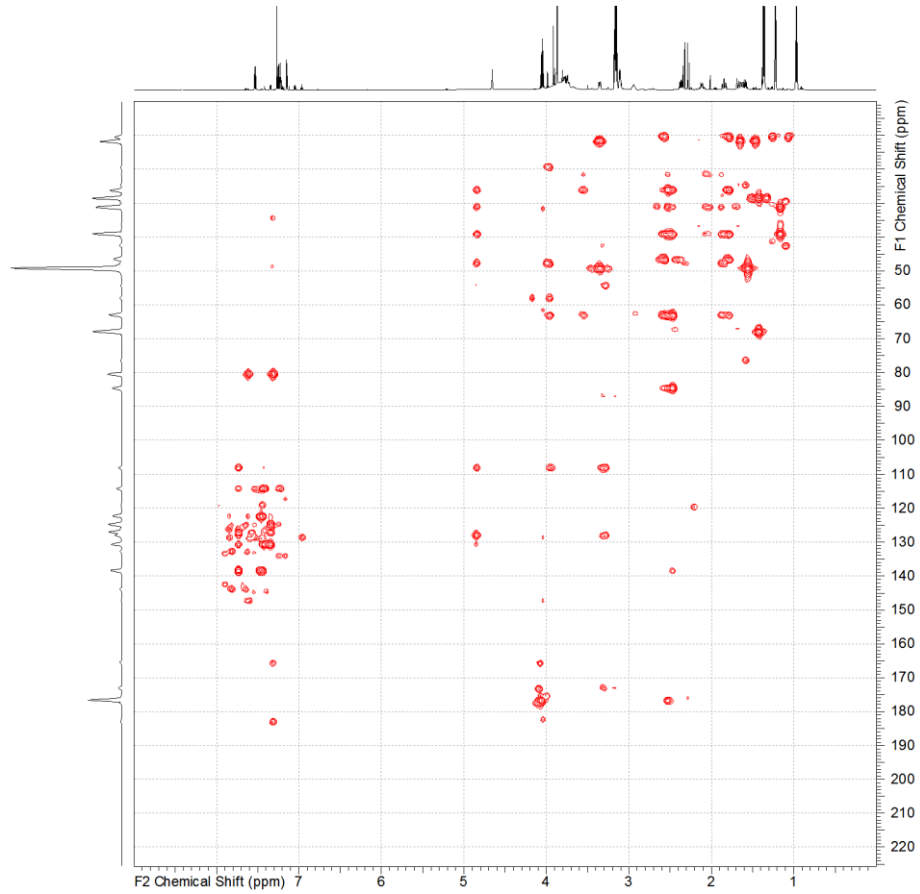
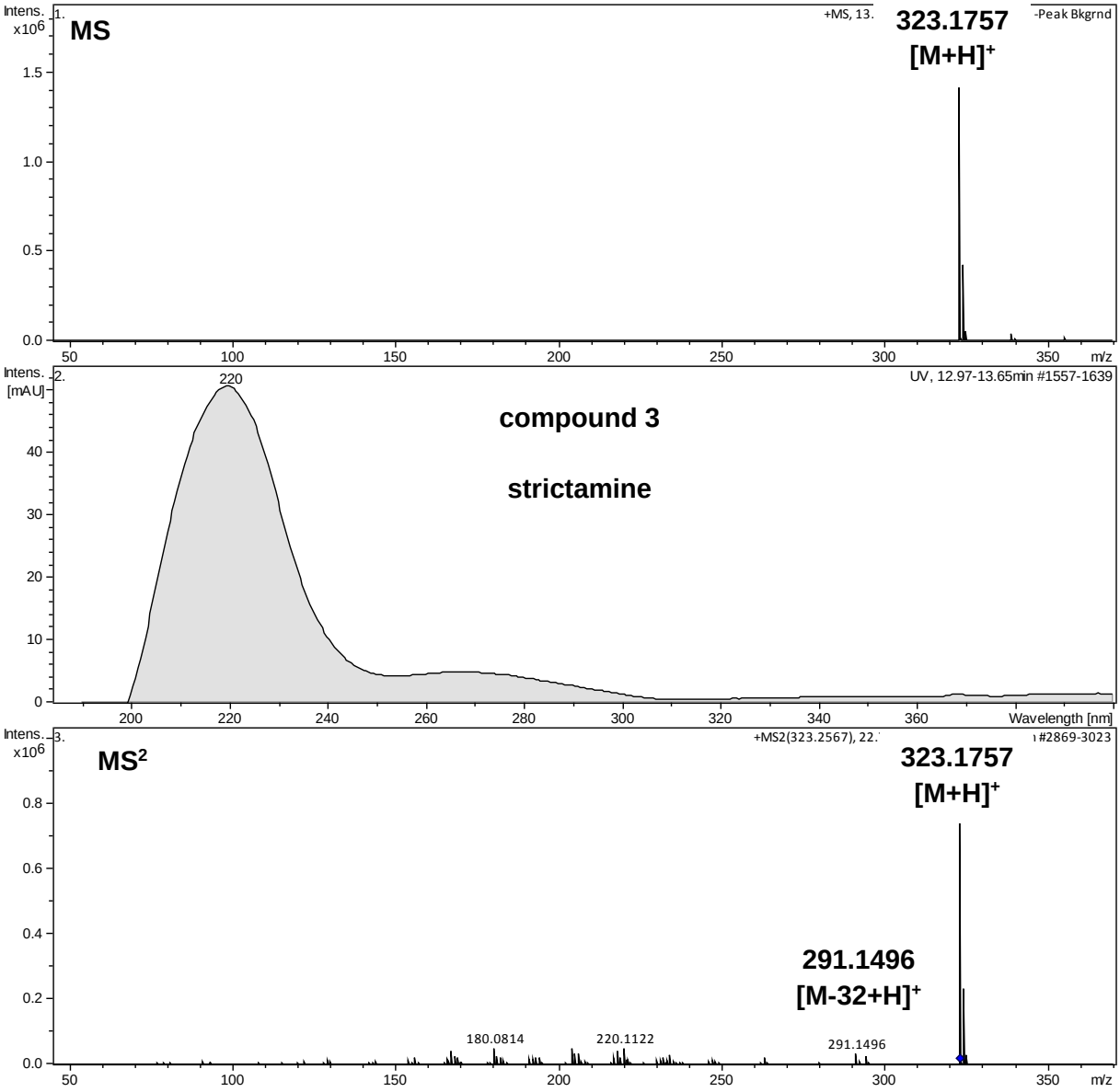


Figure A- 16: ^{1}H-^{13}C-HMBC-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincamine (**2**) xTFA.



Spectrum Data

Meas. m/z	#	Sum Formula	Ion Formula	Adduct	m/z	err [ppm]	mSigma	# mSigma	Score
323.1757	1	$C_{20}H_{22}N_2O_2$	$C_{20}H_{23}N_2O_2$	M+H	323.1754	-1.0	28.1	1	100.00

Figure A- 17: UV, MS and MS-MS spectrum of strictamine (**3**).

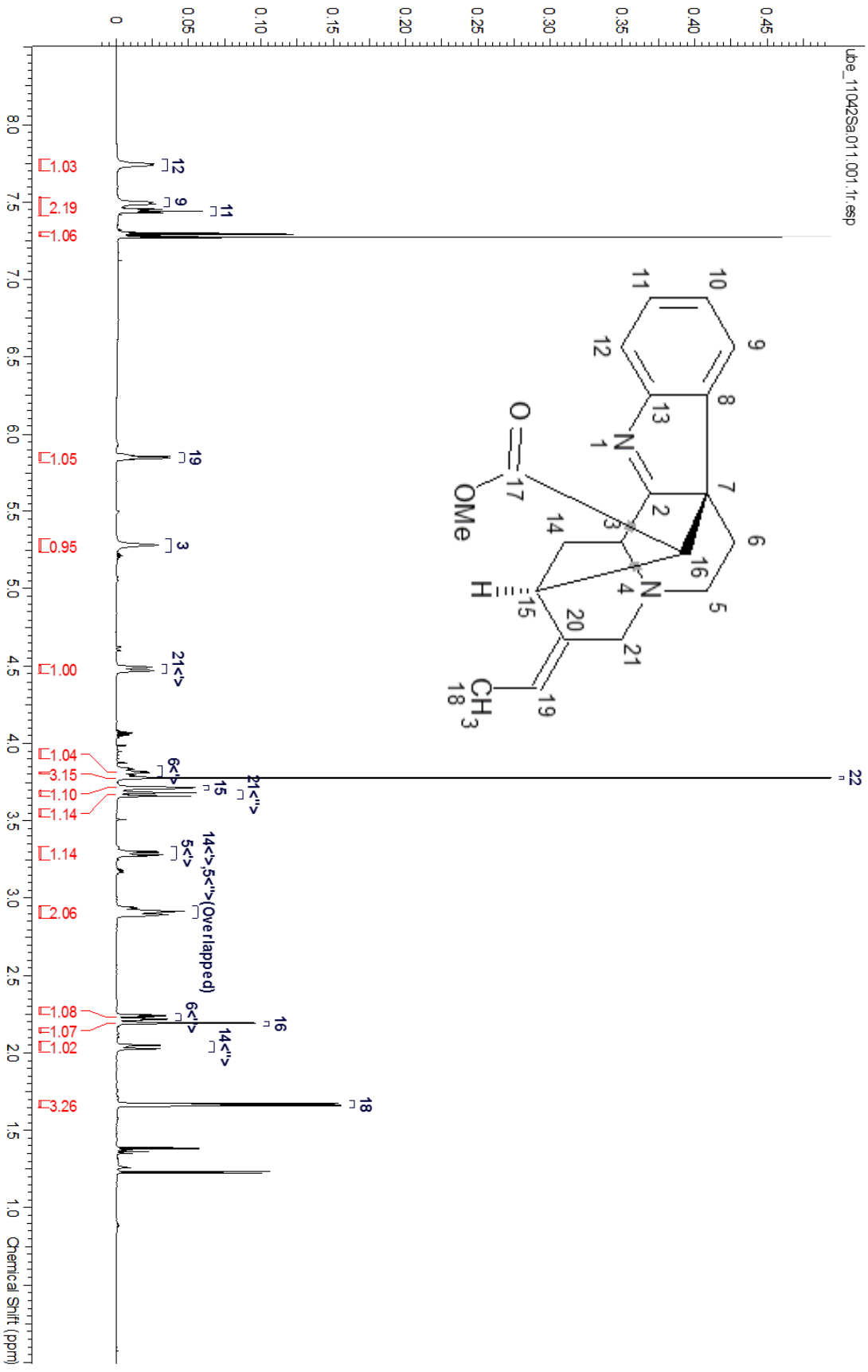


Figure A- 18: ^{1}H-NMR spectrum (700 MHz, $CHCl_3$-*d*) of strictamine (**3**) xTFA.

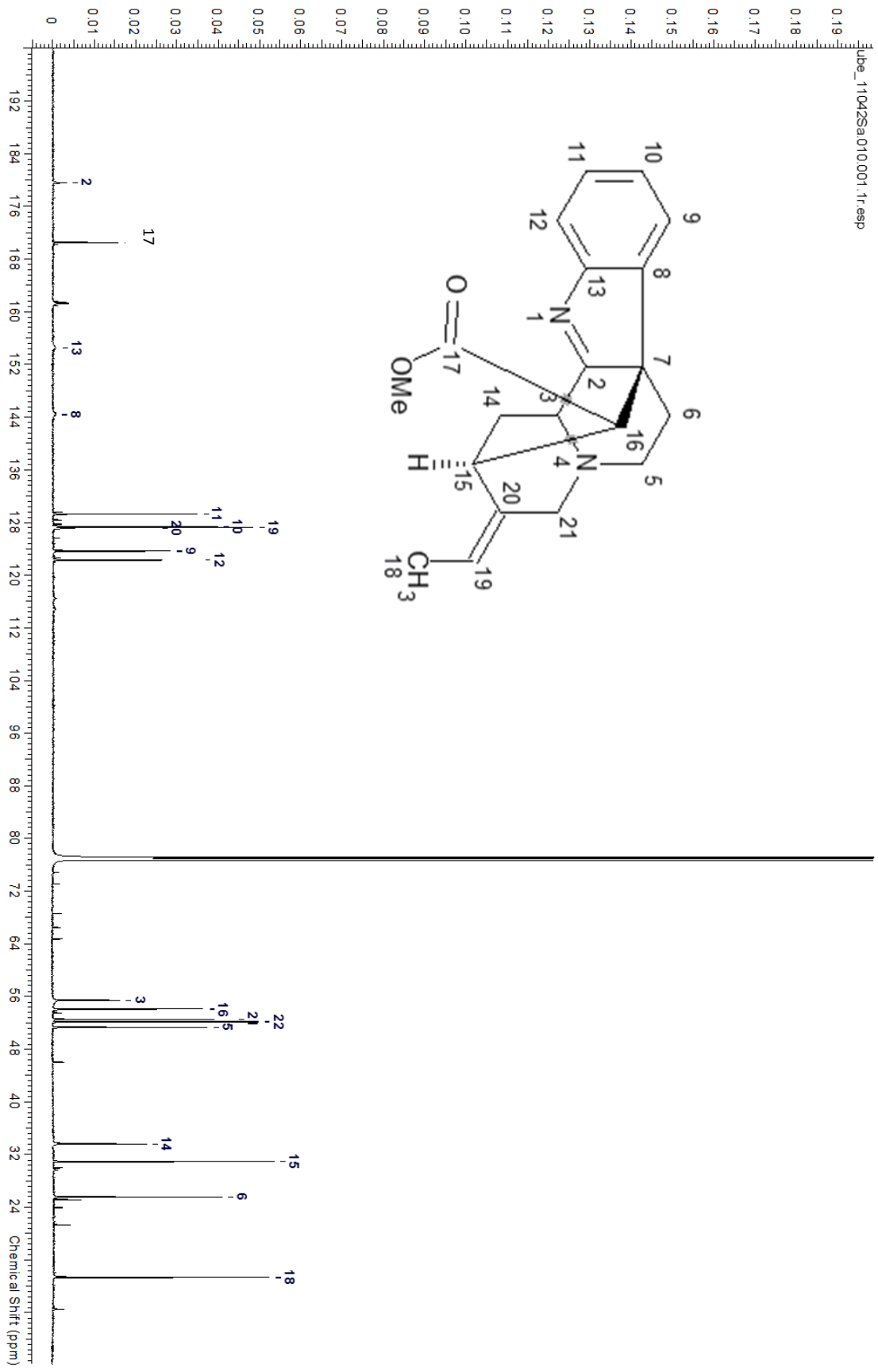


Figure A- 19: ^{13}C-NMR spectrum (175 MHz, $CHCl_3$-*d*) of strictamine (**3**) xTFA.

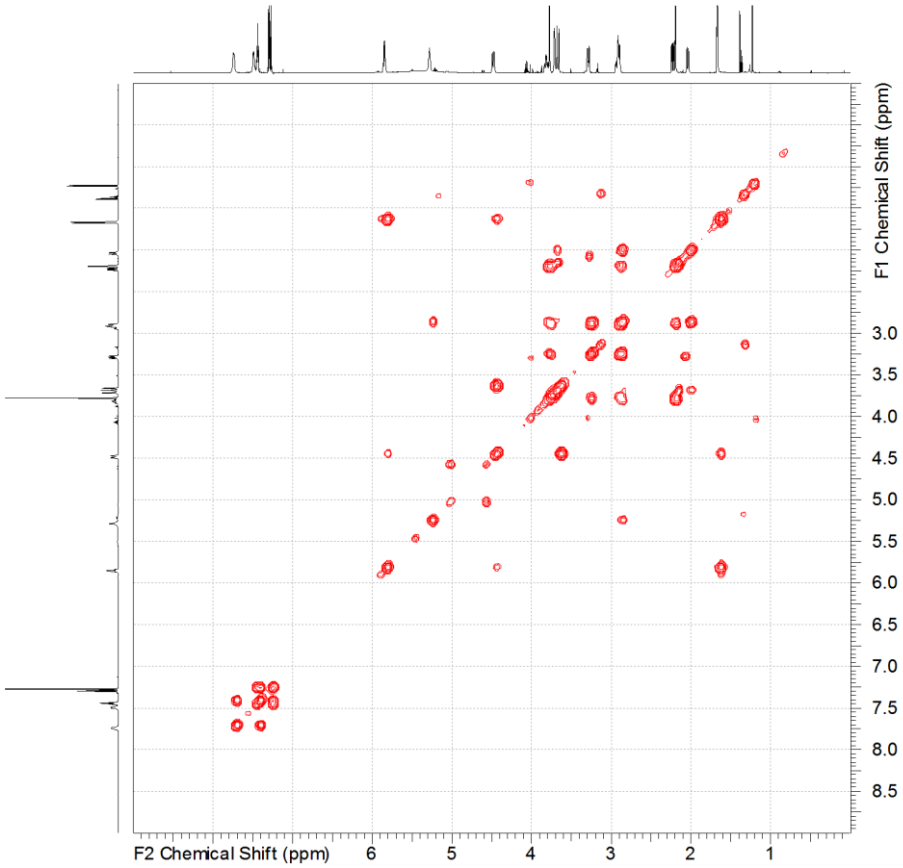


Figure A- 20: ^{1}H-^{1}H-COSY-NMR spectrum (700 MHz, $CHCl_3$-*d*) of strictamine (**3**) xTFA.

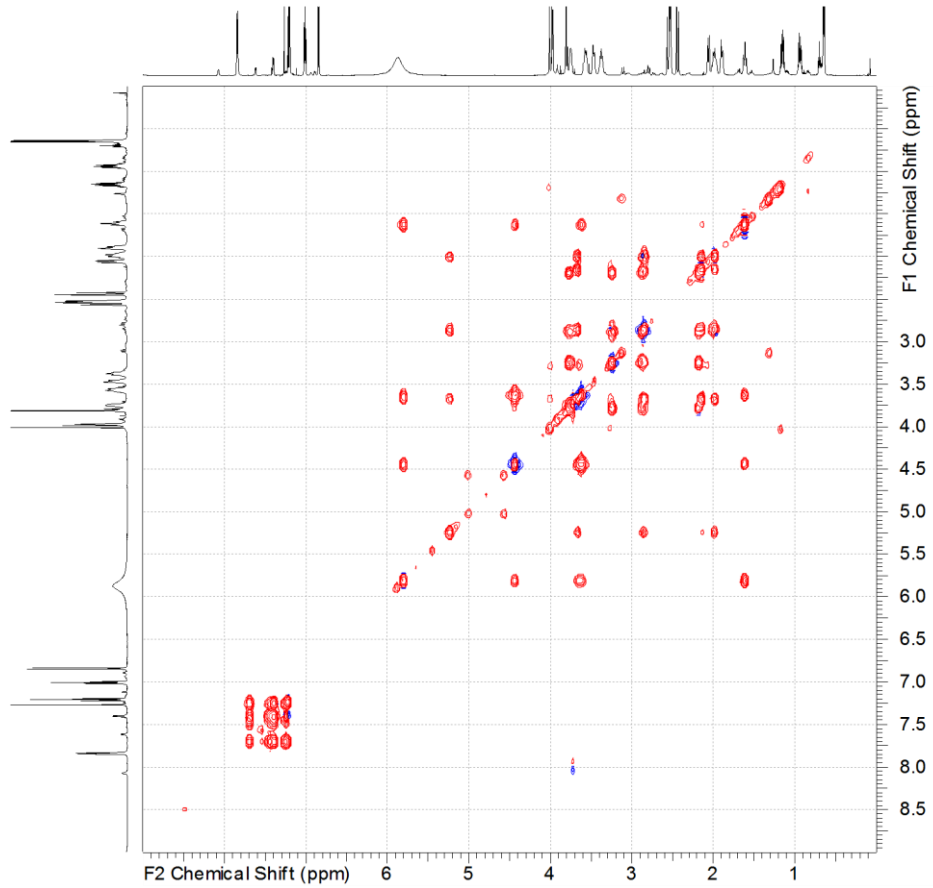


Figure A- 21: ^{1}H-^{1}H-TOCSY-NMR spectrum (700 MHz, $CHCl_3$-*d*) of strictamine (**3**) xTFA.

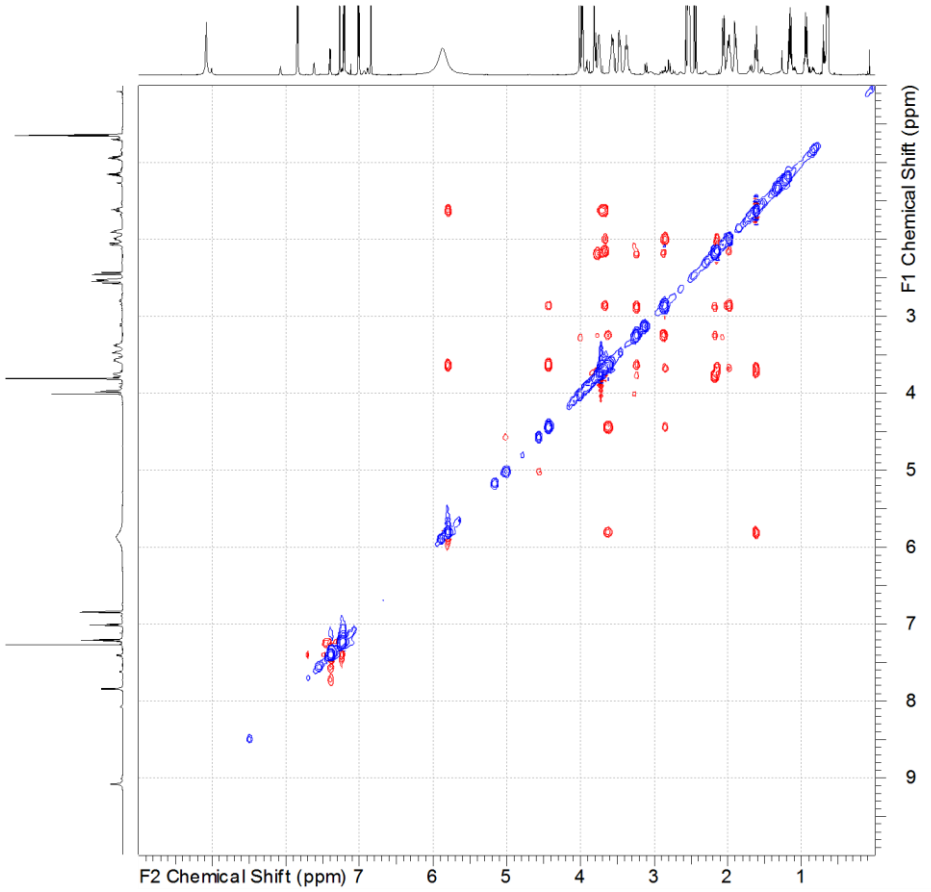


Figure A- 22: ^{1}H-^{1}H-ROESY-NMR spectrum (700 MHz, $CHCl_3$-*d*) of strictamine (**3**) xTFA.

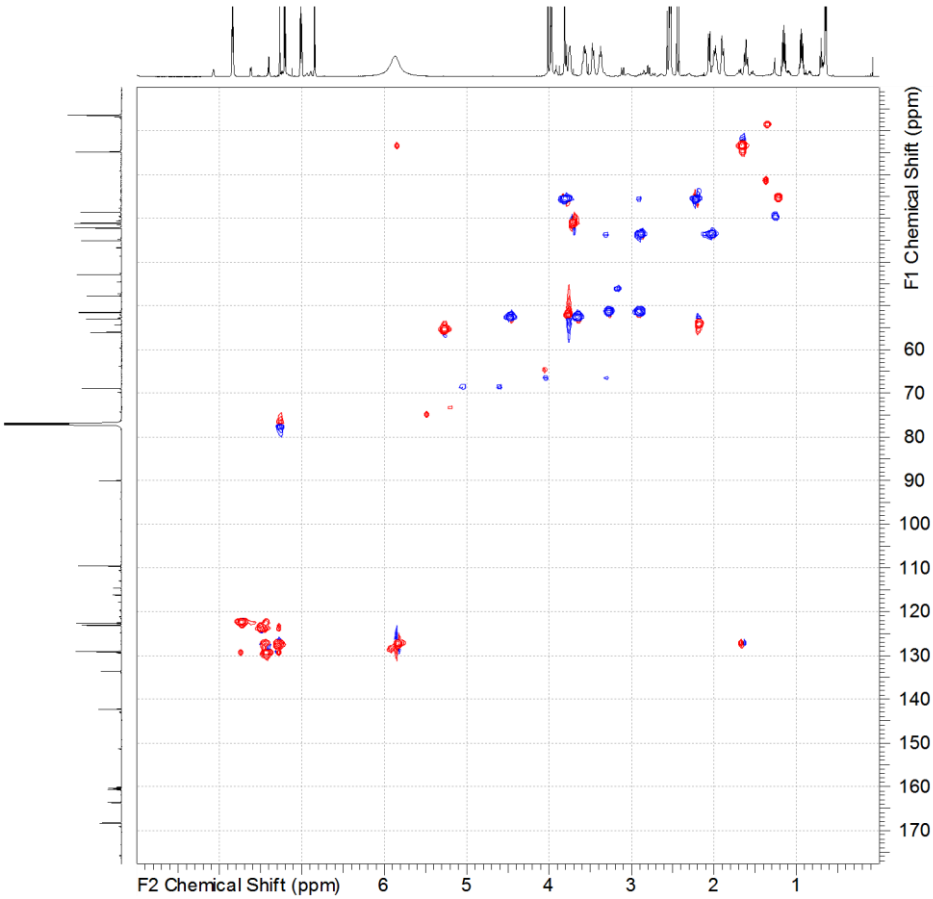


Figure A- 23: ^{1}H-^{13}C-HSQC-DEPT-NMR spectrum (700 MHz, $CHCl_3$-*d*) of strictamine (**3**) xTFA.

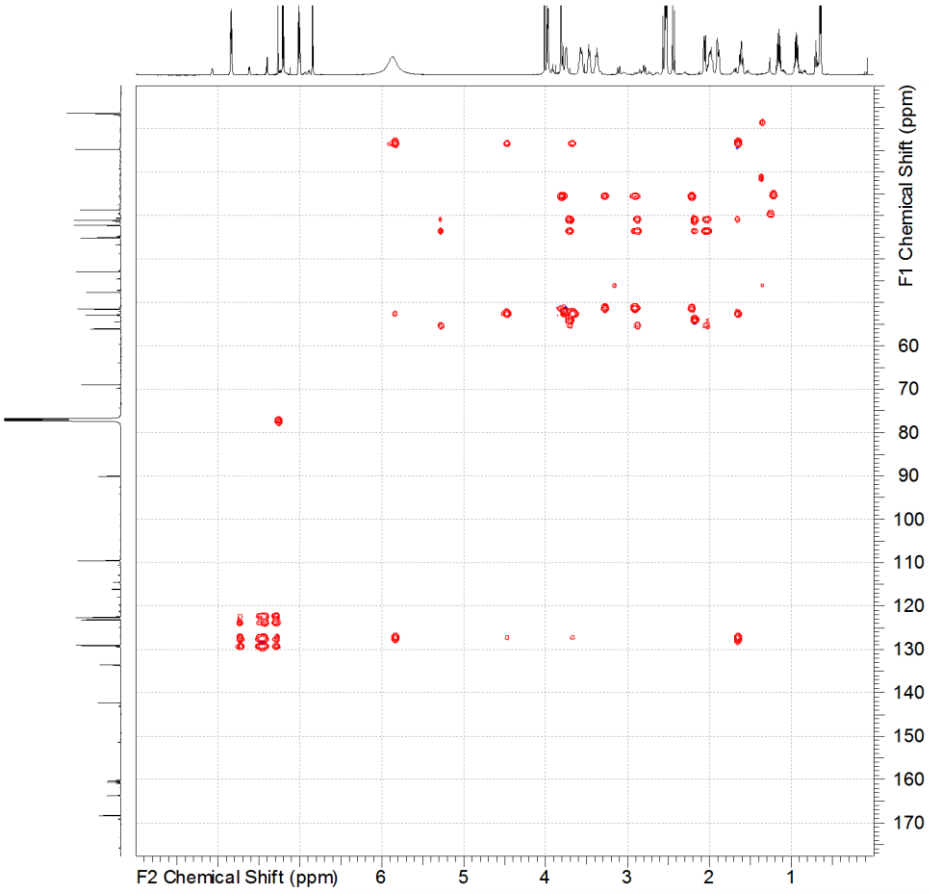


Figure A- 24: ^{1}H-^{13}C-HSQC-NMR spectrum (700 MHz, $CHCl_3$-*d*) of strictamine (**3**) xTFA.

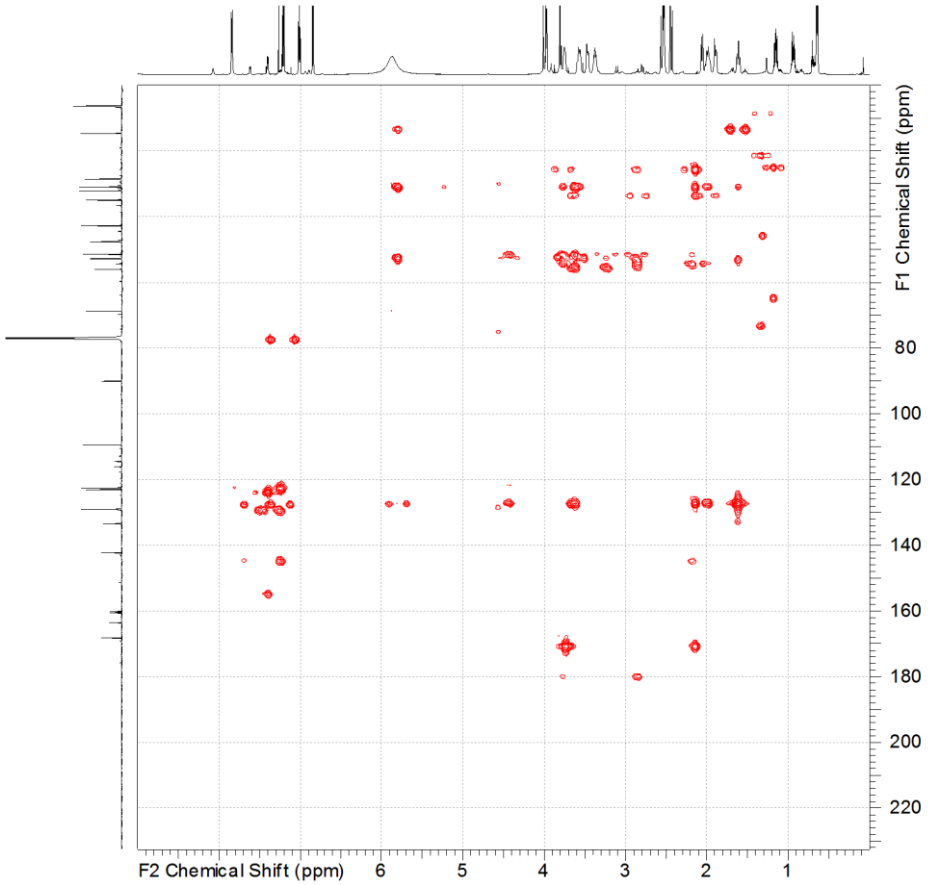
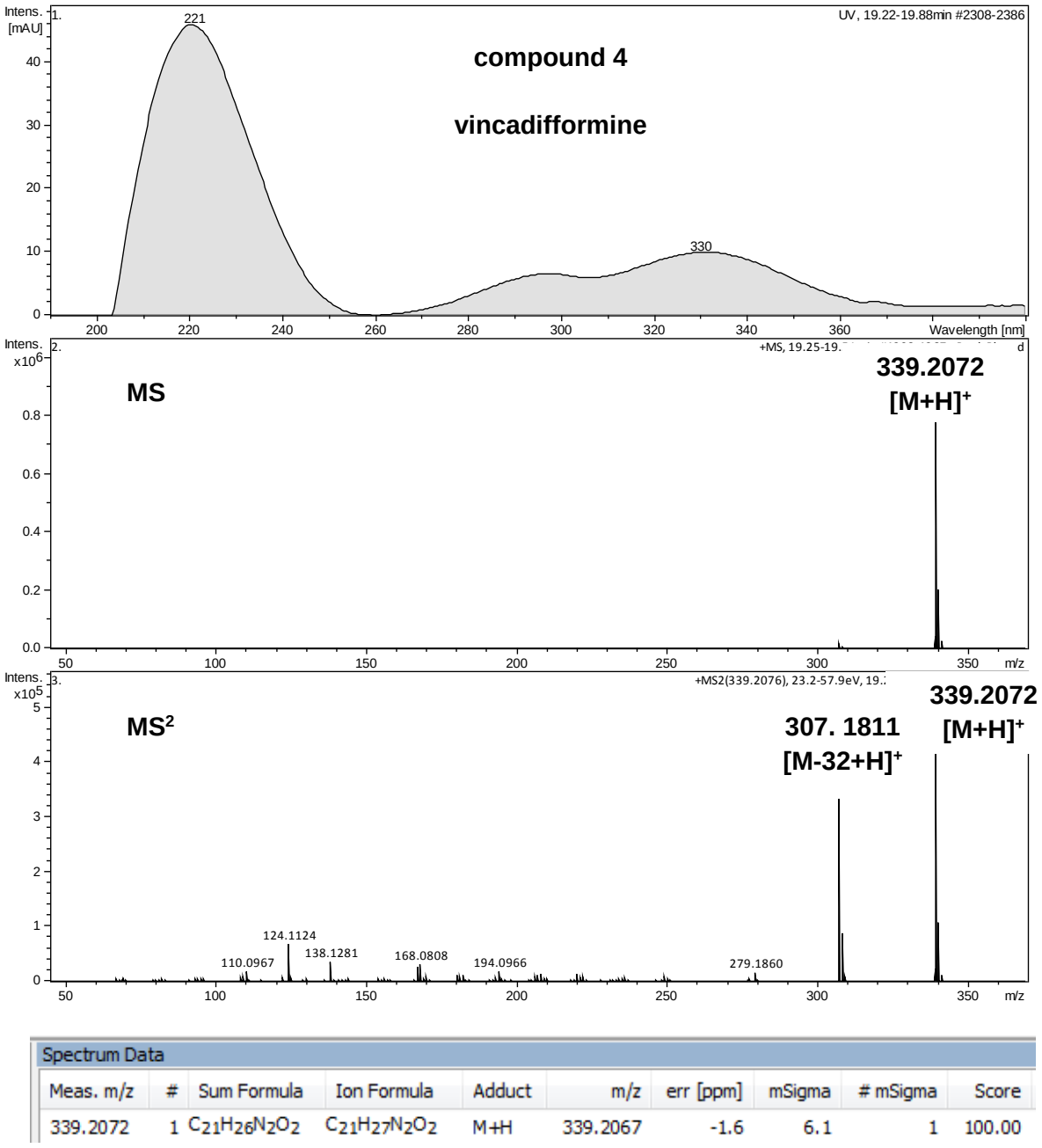


Figure A- 25: ^{1}H-^{13}C-HMBC-NMR spectrum (700 MHz, $CHCl_3$-*d*) of strictamine (**3**) xTFA.



Spectrum Data

Meas. m/z	#	Sum Formula	Ion Formula	Adduct	m/z	err [ppm]	mSigma	# mSigma	Score
339.2072	1	$C_{21}H_{26}N_2O_2$	$C_{21}H_{27}N_2O_2$	M+H	339.2067	-1.6	6.1	1	100.00

Figure A- 26:. UV, MS and MS-MS spectrum of vincadifformine (**4**).

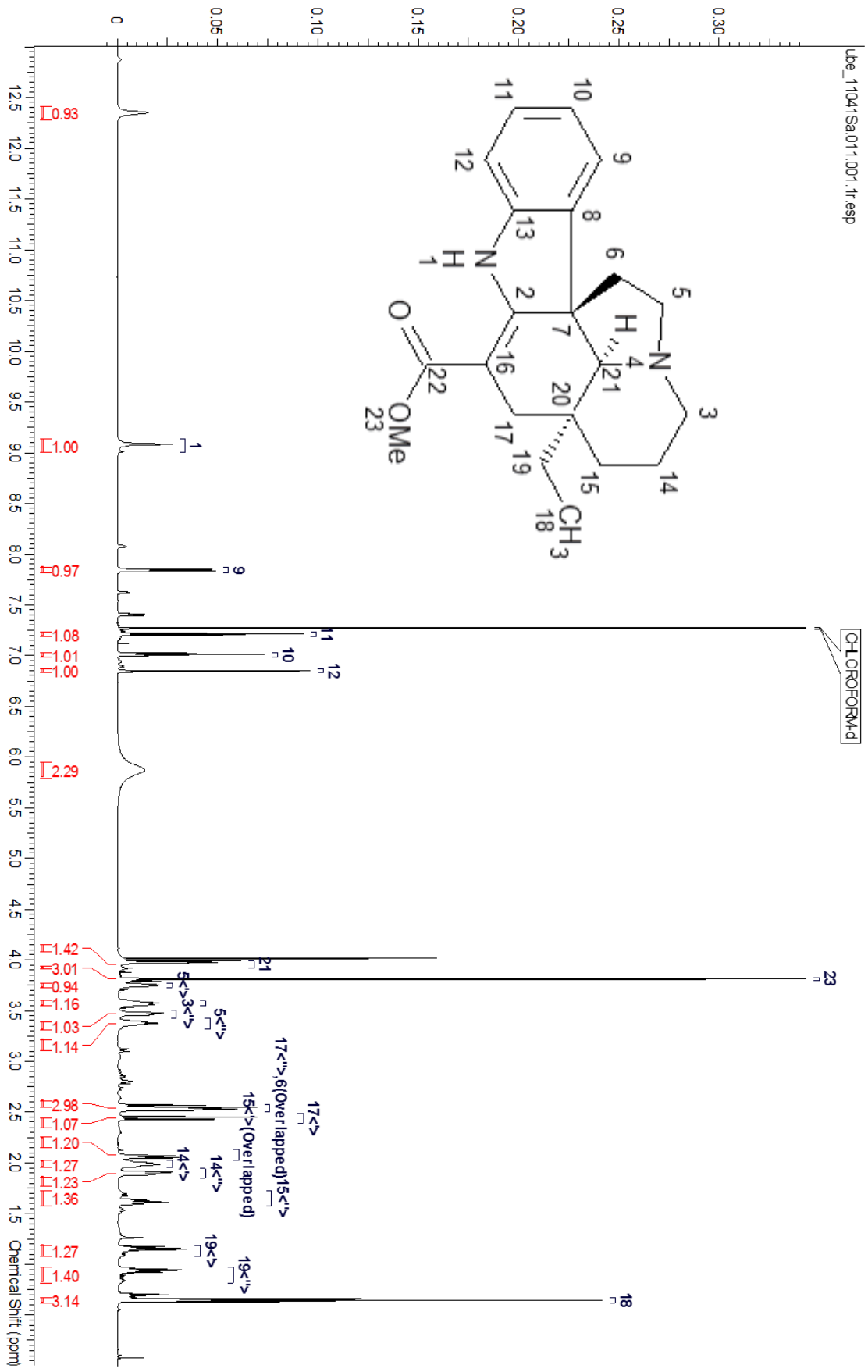


Figure A- 27: ^{1}H NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincadifformine (**4**) xTFA.

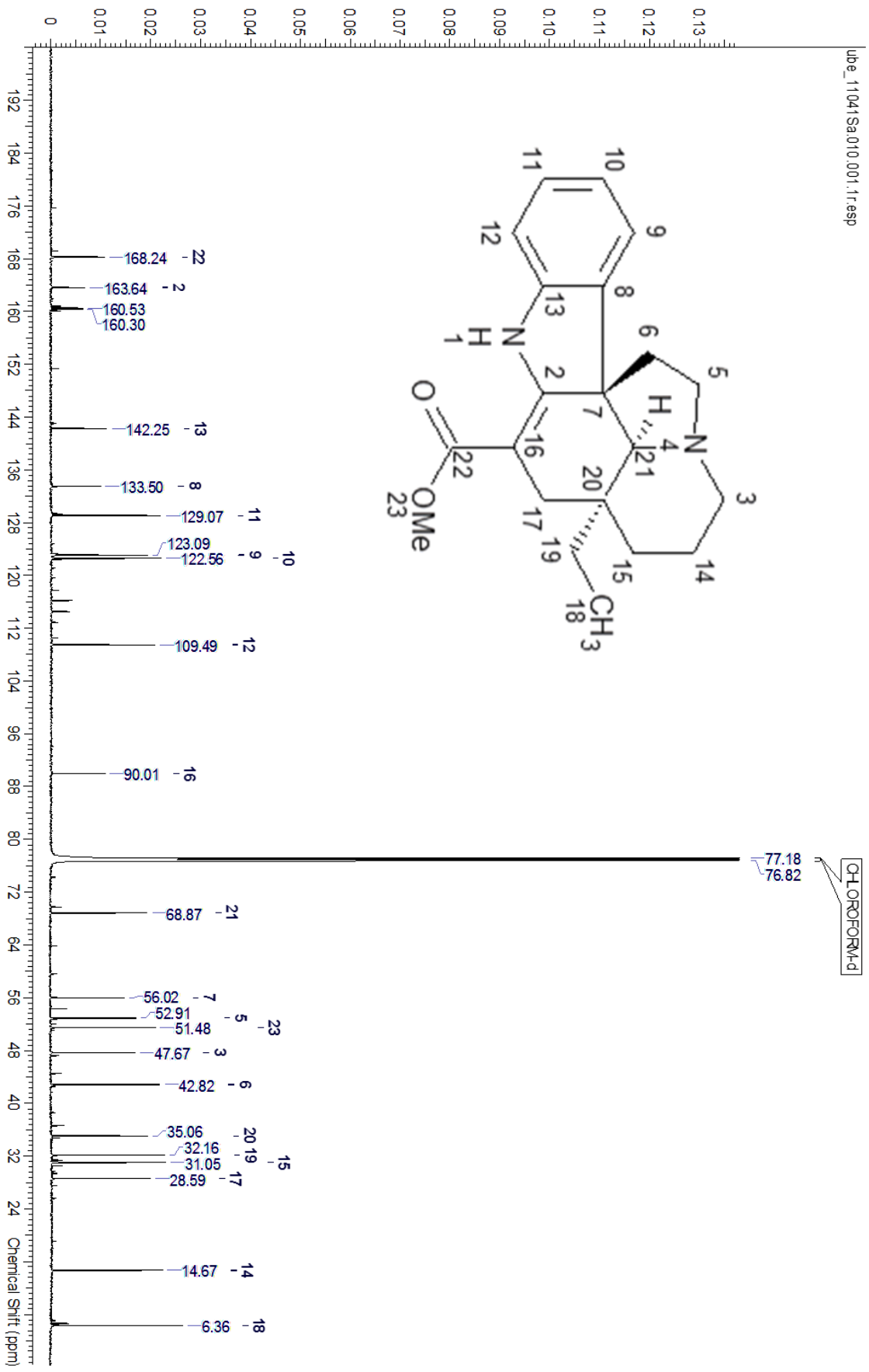


Figure A- 28: ^{13}C NMR spectrum (175 MHz, $CHCl_3$-*d*) of vincadifformine (**4**) xTFA.

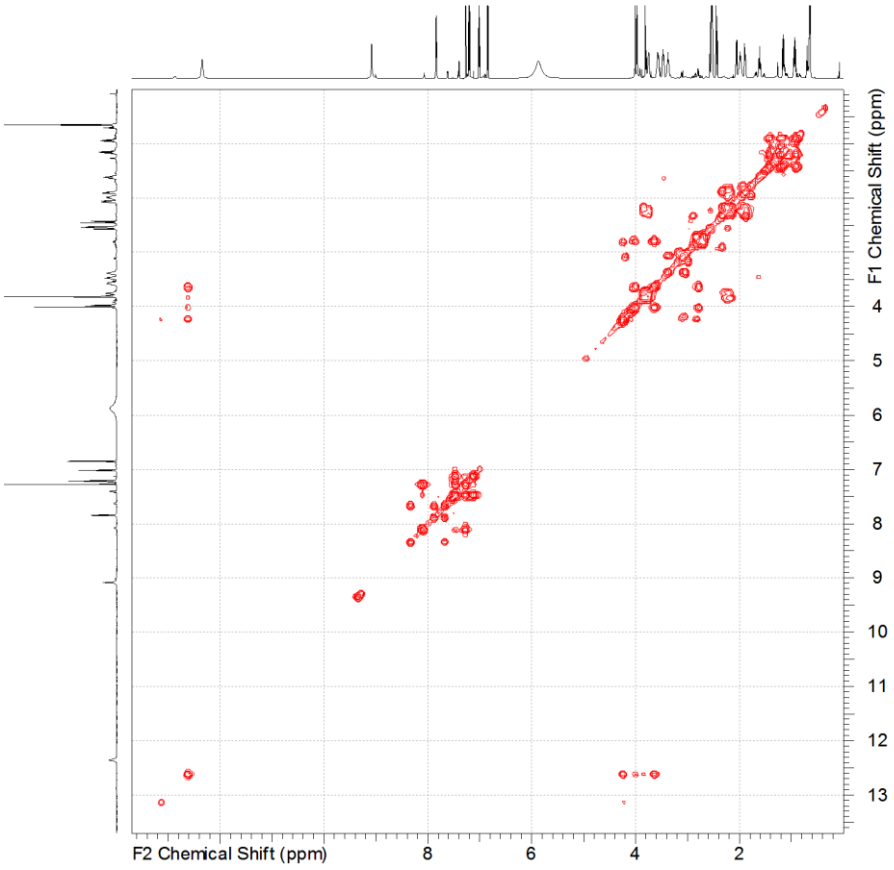


Figure A- 29: ^{1}H-^{1}H-COSY-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincadifformine (**4**) xTFA.

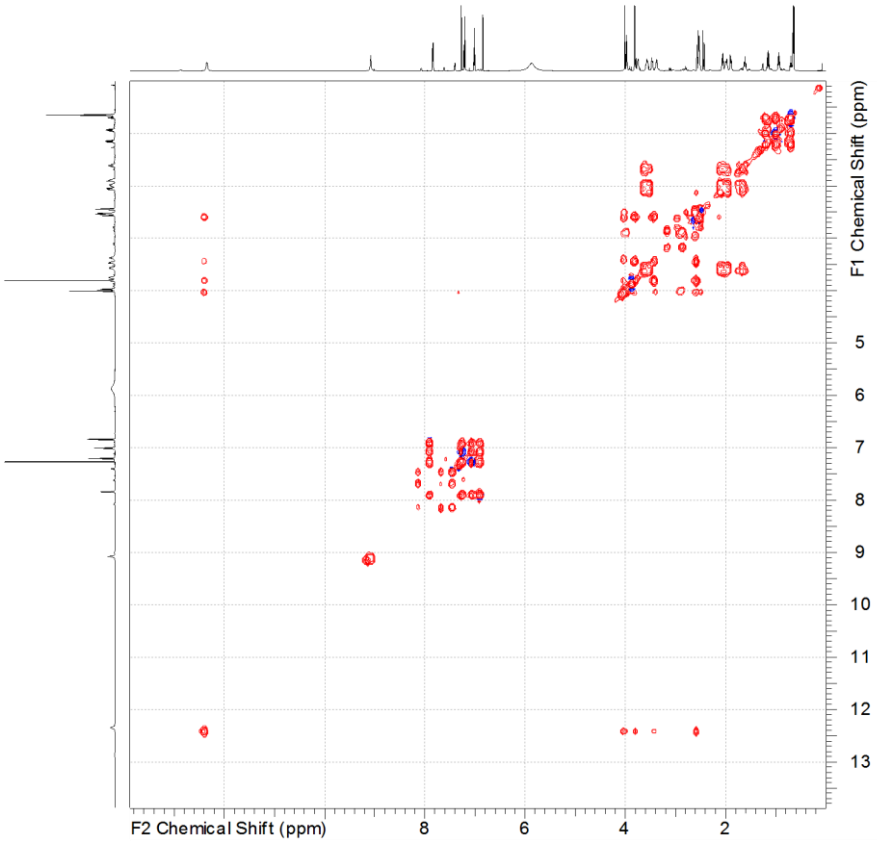


Figure A- 30: ^{1}H-^{1}H-TOCSY-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincadifformine (**4**) xTFA.

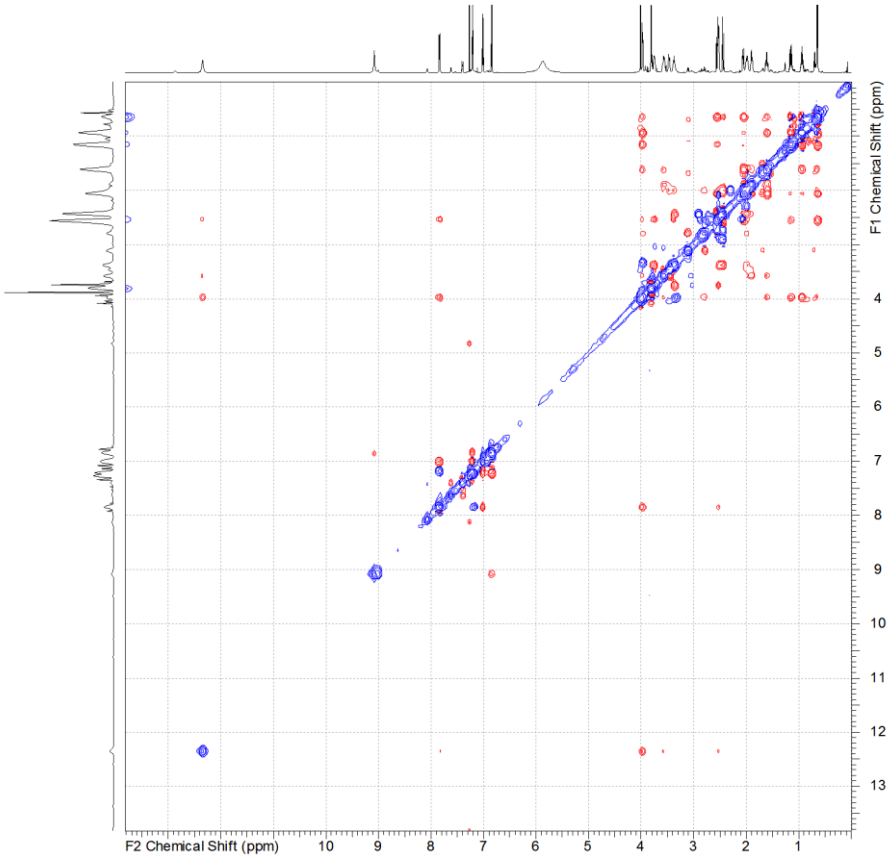


Figure A- 31: ^{1}H-^{1}H-ROESY-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincadifformine (**4**) xTFA.

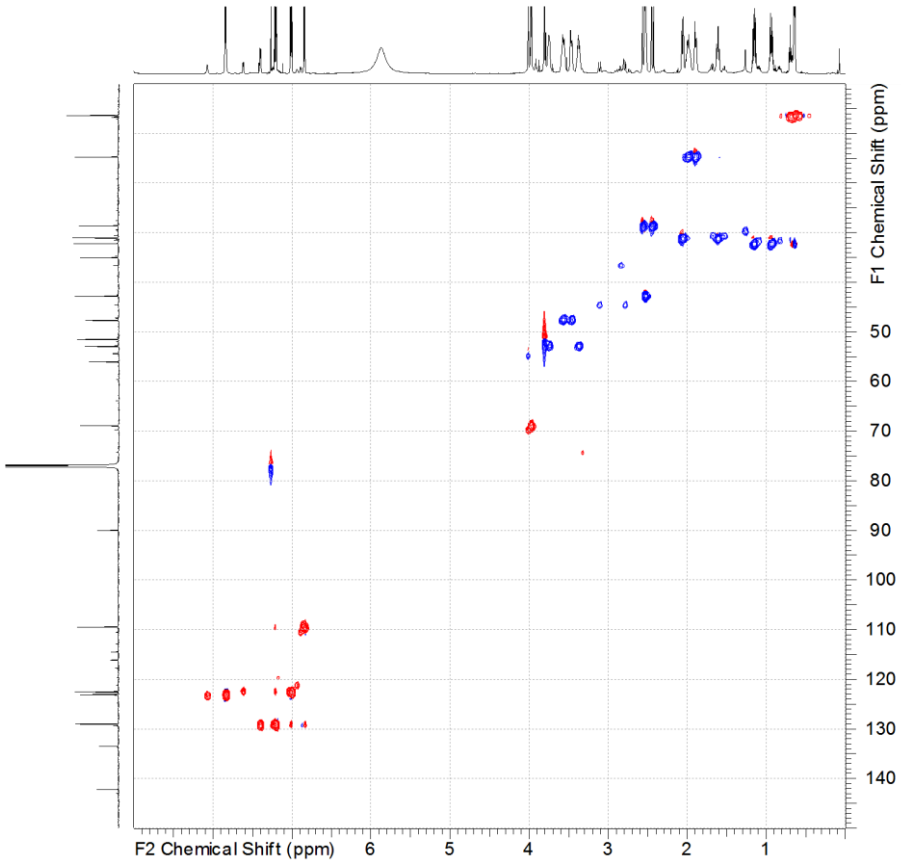


Figure A- 32: ^{1}H-^{13}C-HSQC-DEPT-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincadifformine (**4**) xTFA.

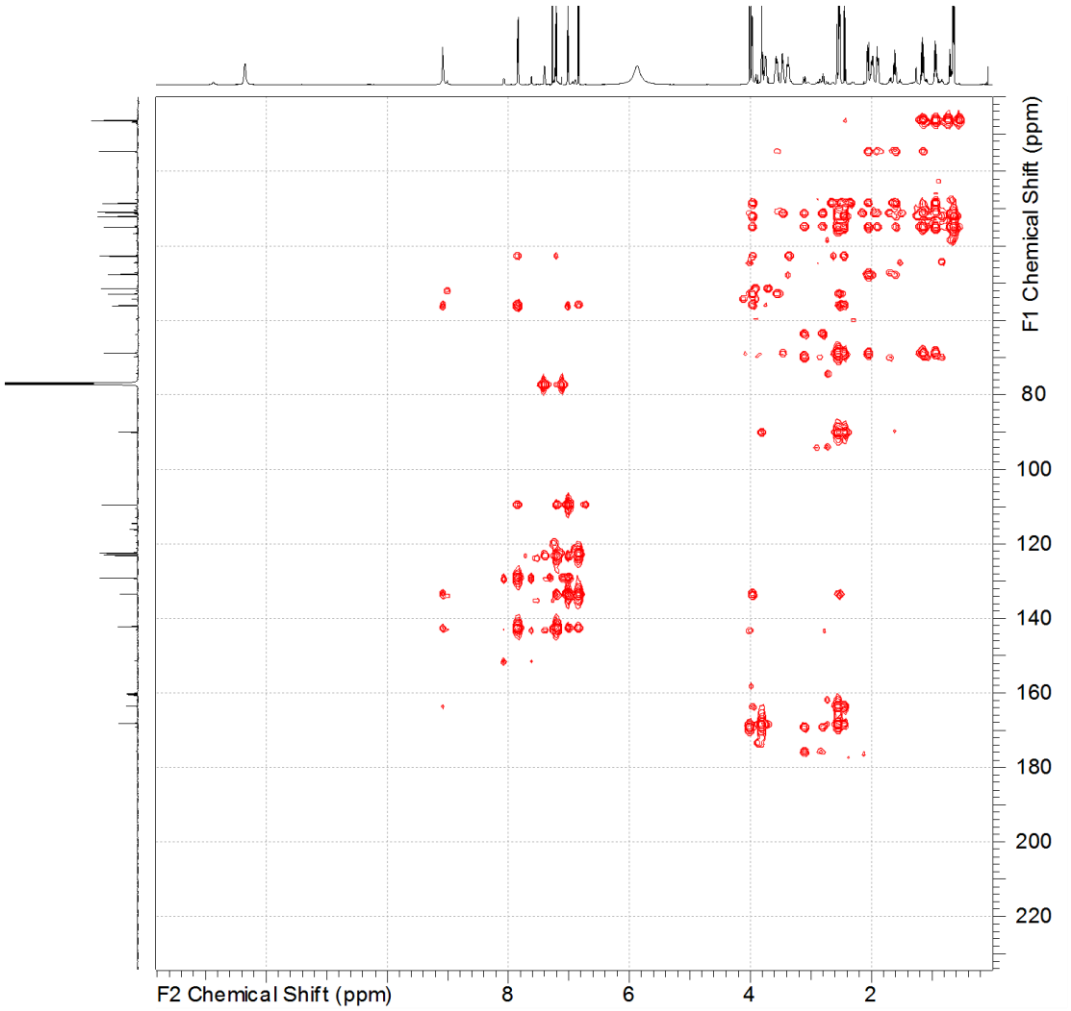
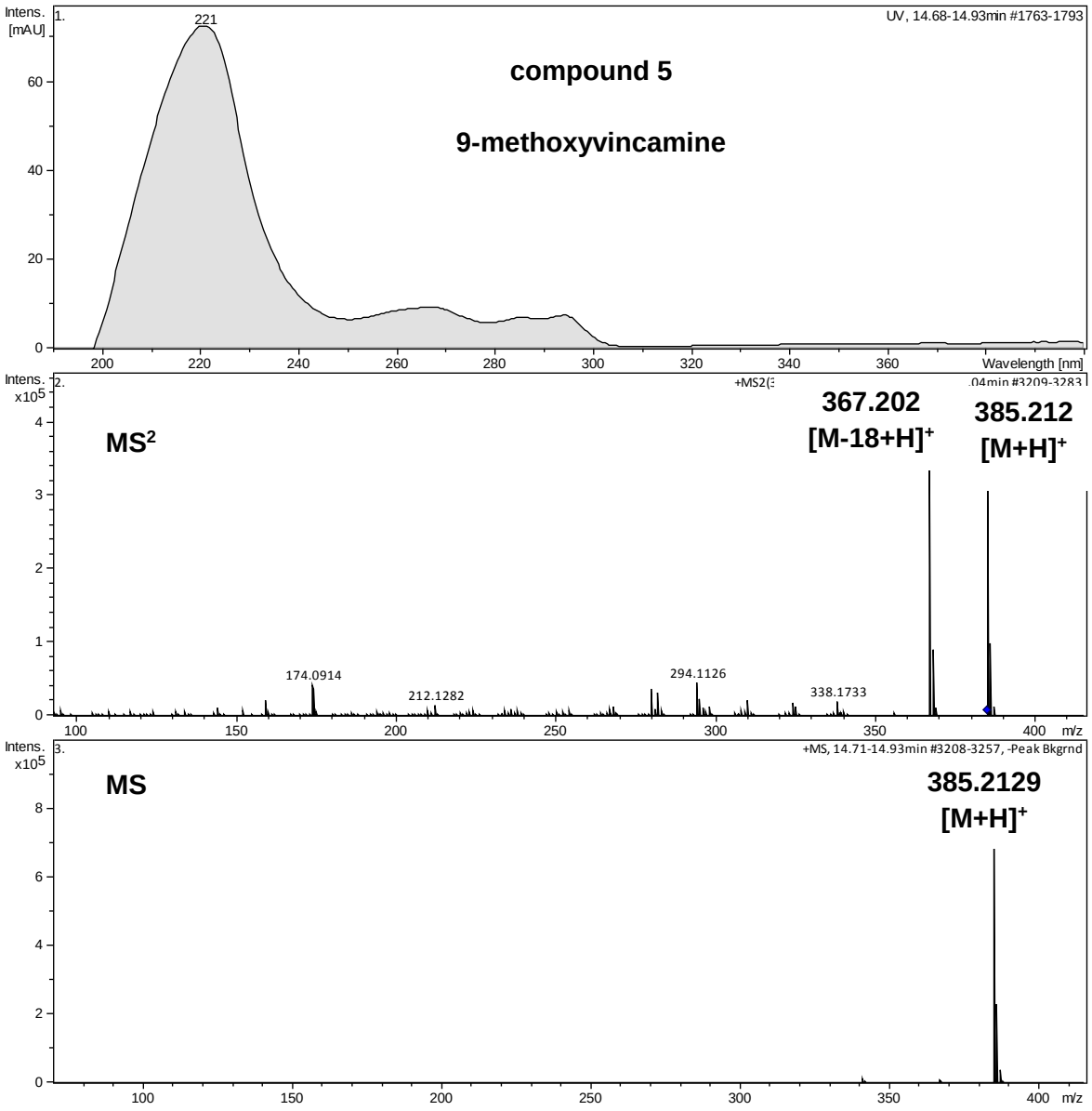


Figure A- 33: ^{1}H-^{13}C-HMBC-NMR spectrum (700 MHz, $CHCl_3$-*d*) of vincadifformine (**4**) xTFA.



Spectrum Data

Meas. m/z	#	Sum Formula	Ion Formula	Adduct	m/z	err [ppm]	mSigma	# mSigma	Score
385.2130	1	$C_{23}H_{24}N_6$	$C_{23}H_{25}N_6$	M+H	385.2135	1.4	4.8	1	100.00
	2	$C_{22}H_{28}N_2O_4$	$C_{22}H_{29}N_2O_4$	M+H	385.2122	-2.1	16.4	2	69.55

Figure A- 34: UV, MS and: MS-MS spectrum of 9-methoxyvincamine (**5**).

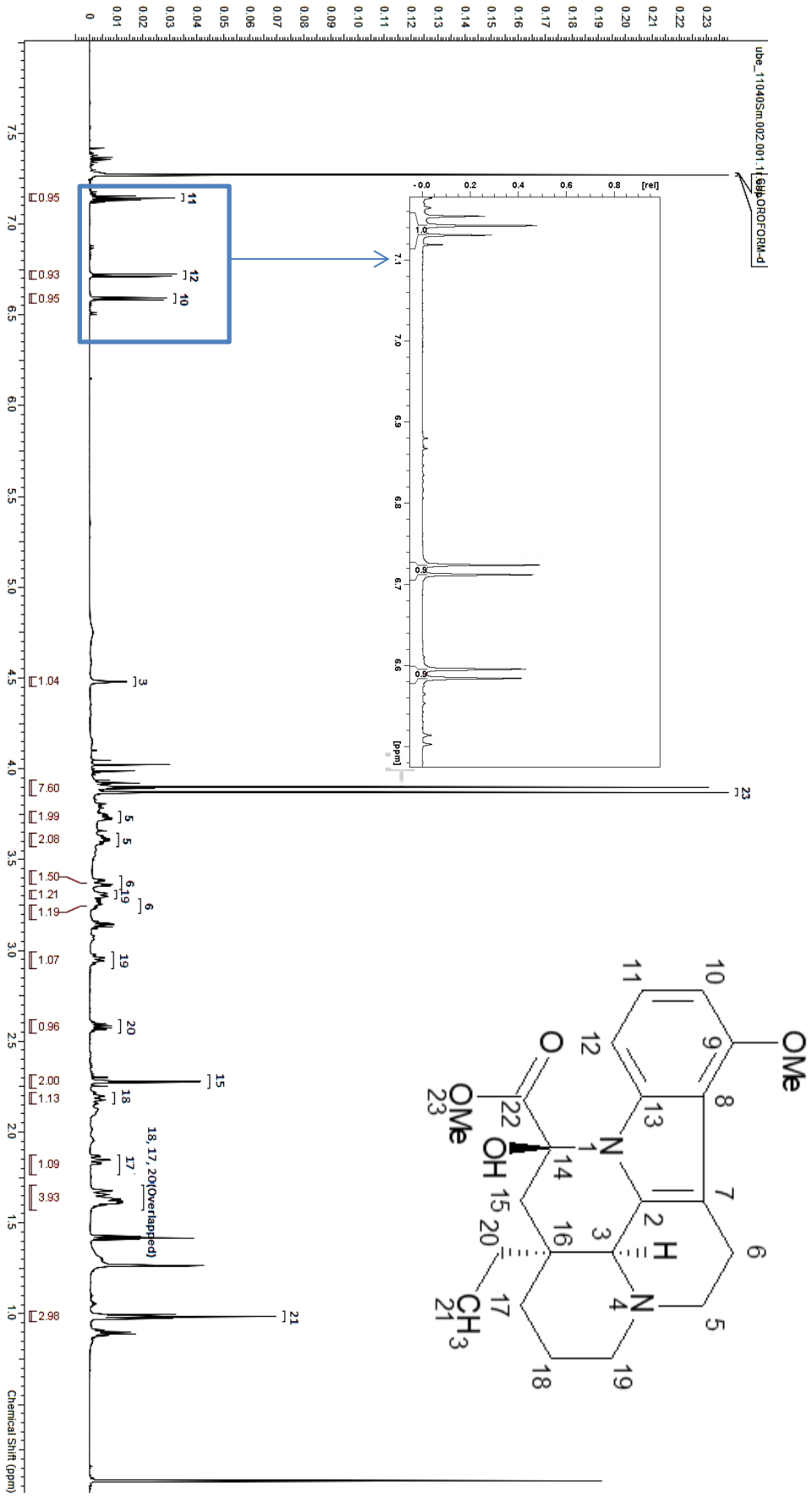


Figure A- 35: ^{1}H- NMR spectrum (700MHz, $CHCl_3$-*d*) of 9-methoxyvincamine (**5**) xTFA.

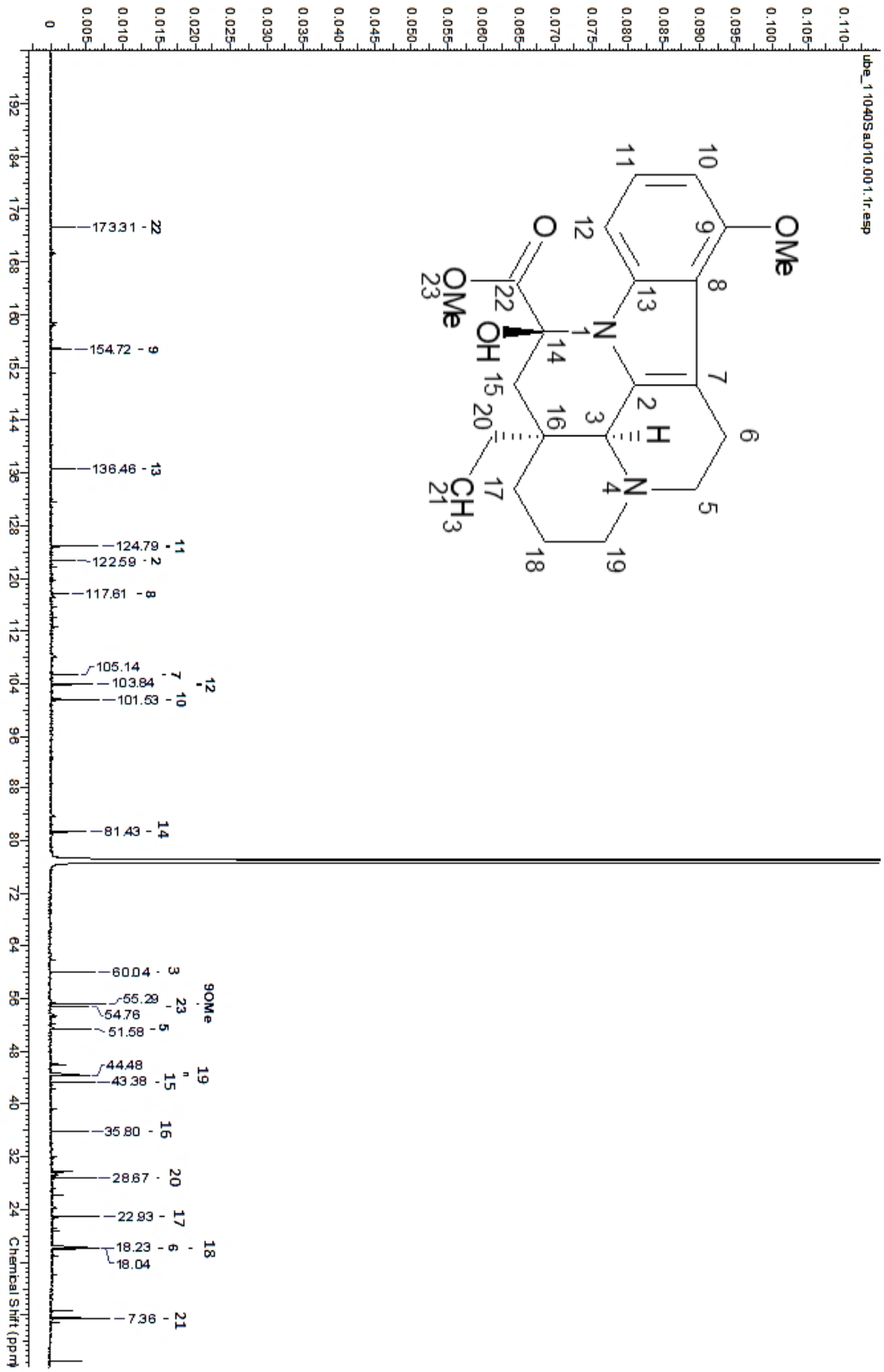


Figure A- 36: ^{13}C-NMR spectrum (175MHz, $CHCl_3$-*d*) of 9-methoxyvincamine (**5**) xTFA.

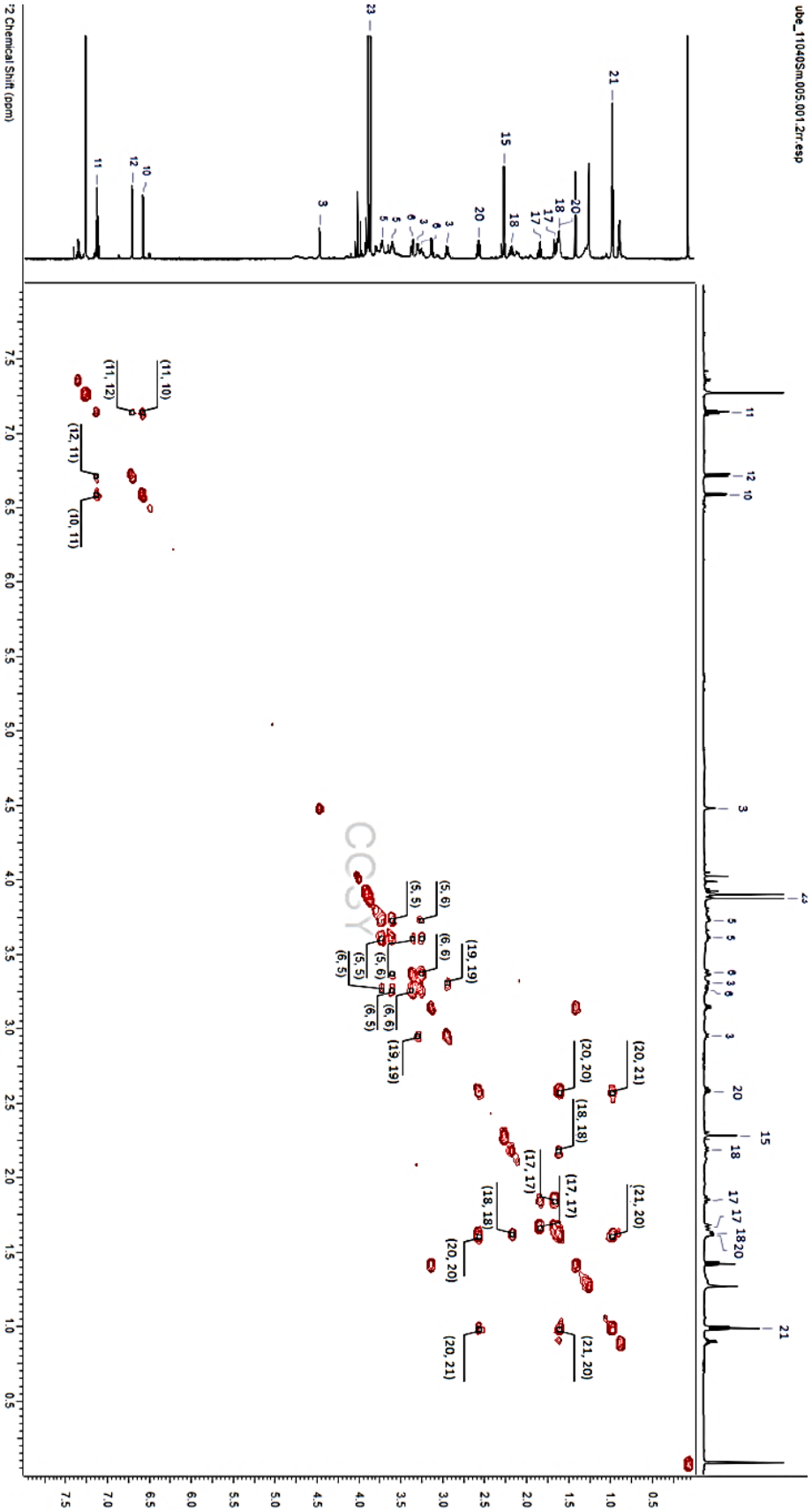


Figure A- 37: ^{1}H-^{1}H-COSY NMR spectrum (700 MHz, $CHCl_3$-*d*) of 9-methoxyvincamine (**5**) xTFA.

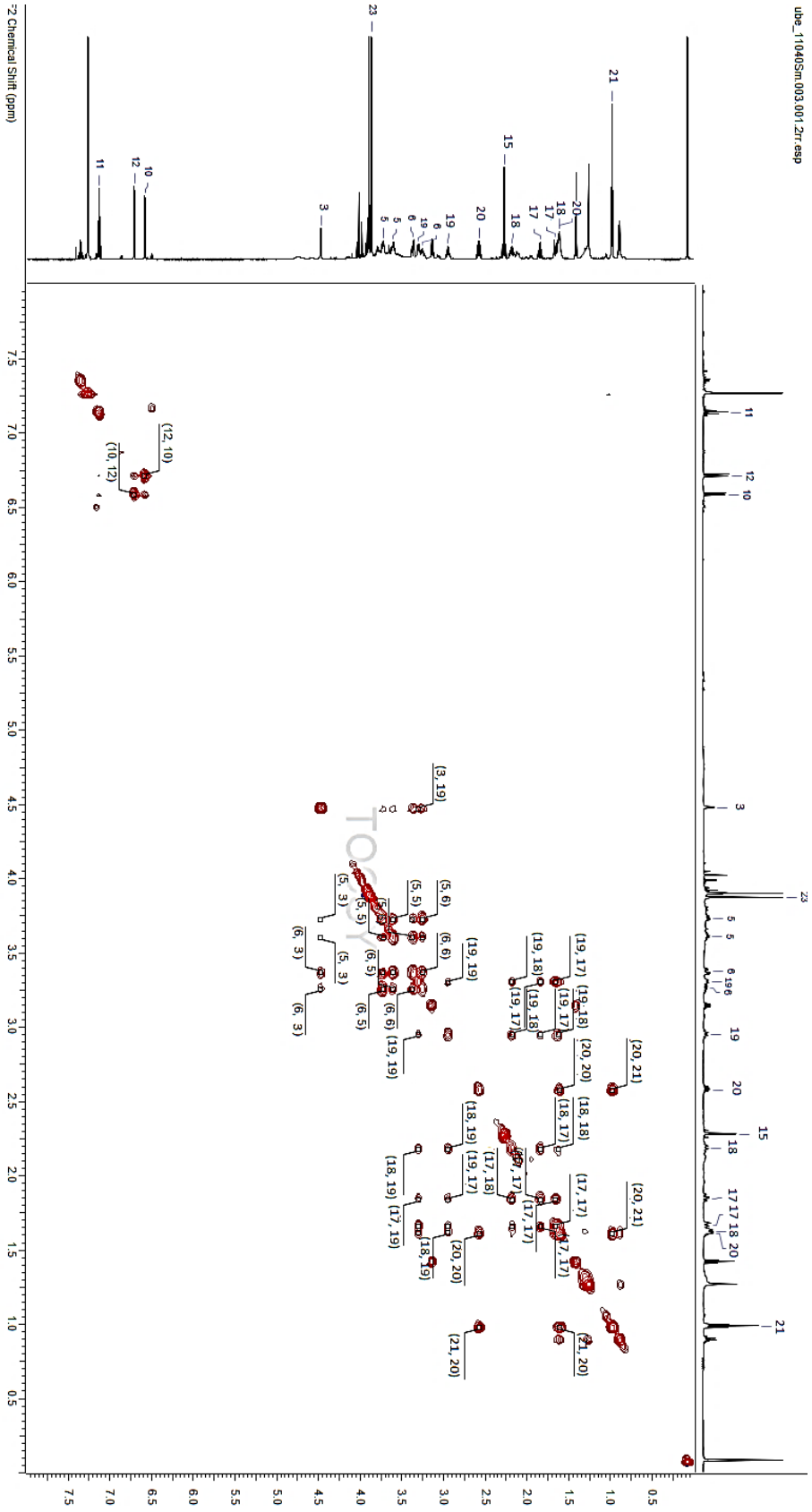


Figure A- 38: ^{1}H-^{1}H-TOCSY NMR spectrum (700 MHz, $CHCl_3$-*d*) of 9-methoxyvincamine (**5**) xTFA**.**

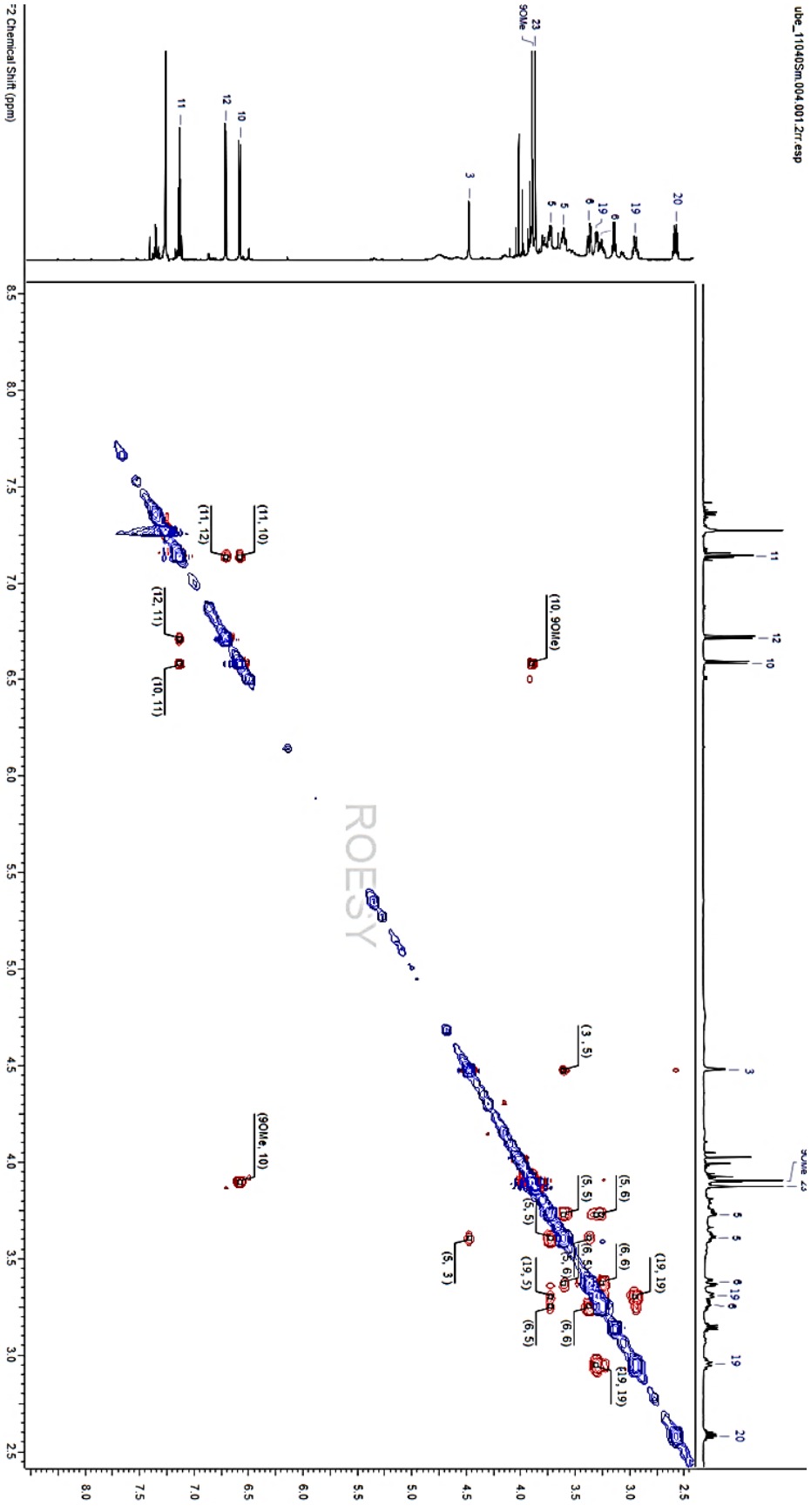


Figure A- 39: ^{1}H-^{1}H-ROESY NMR spectrum (700 MHz, $CHCl_3$-*d*) of 9-methoxyvincamine (**5**) xTFA.

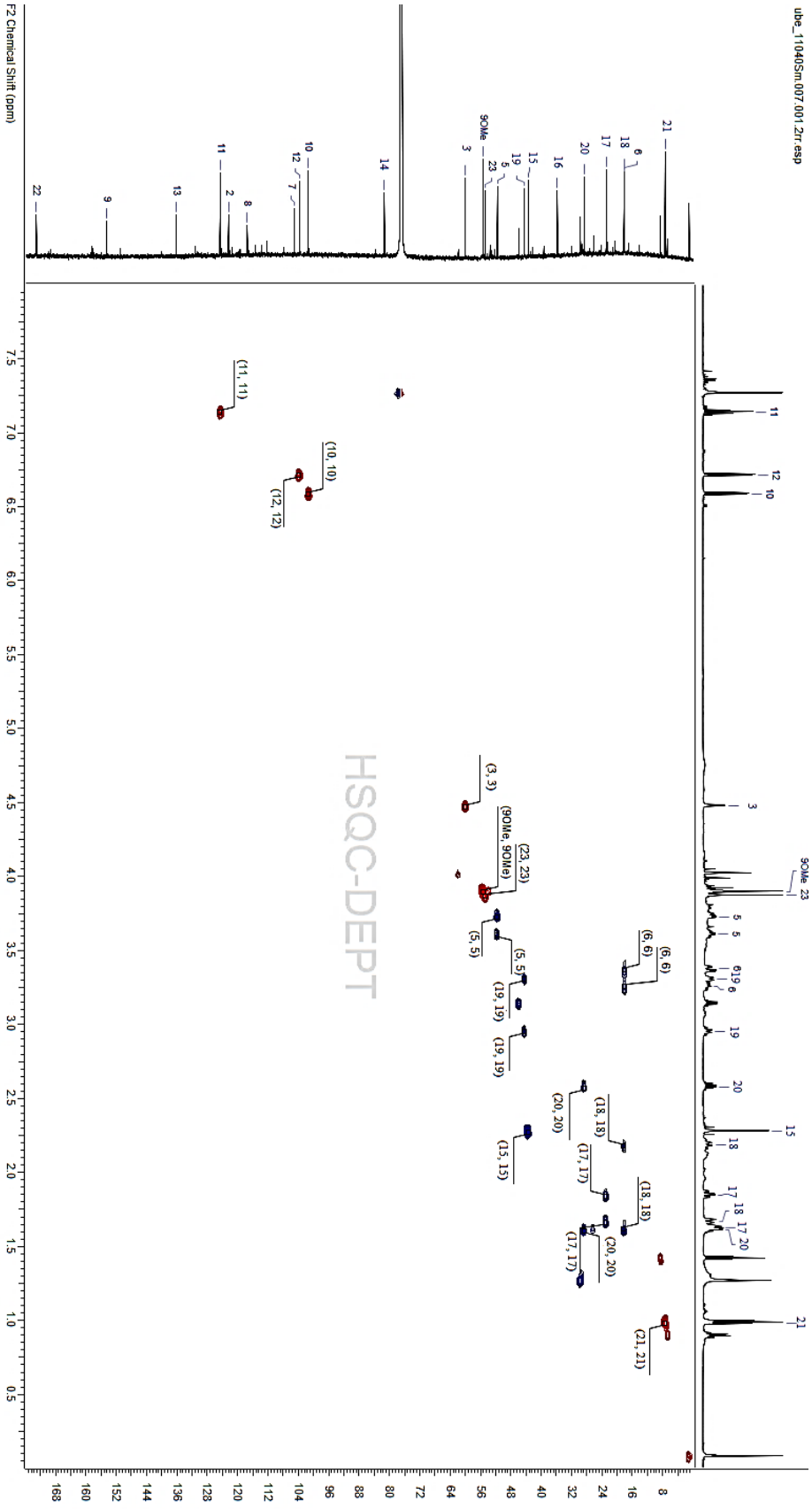


Figure A- 40: ^{1}H-^{13}C-HSQC-NMR spectrum (700 MHz, $CHCl_3$-*d*) of 9-methoxyvincamine (**5**) xTFA.

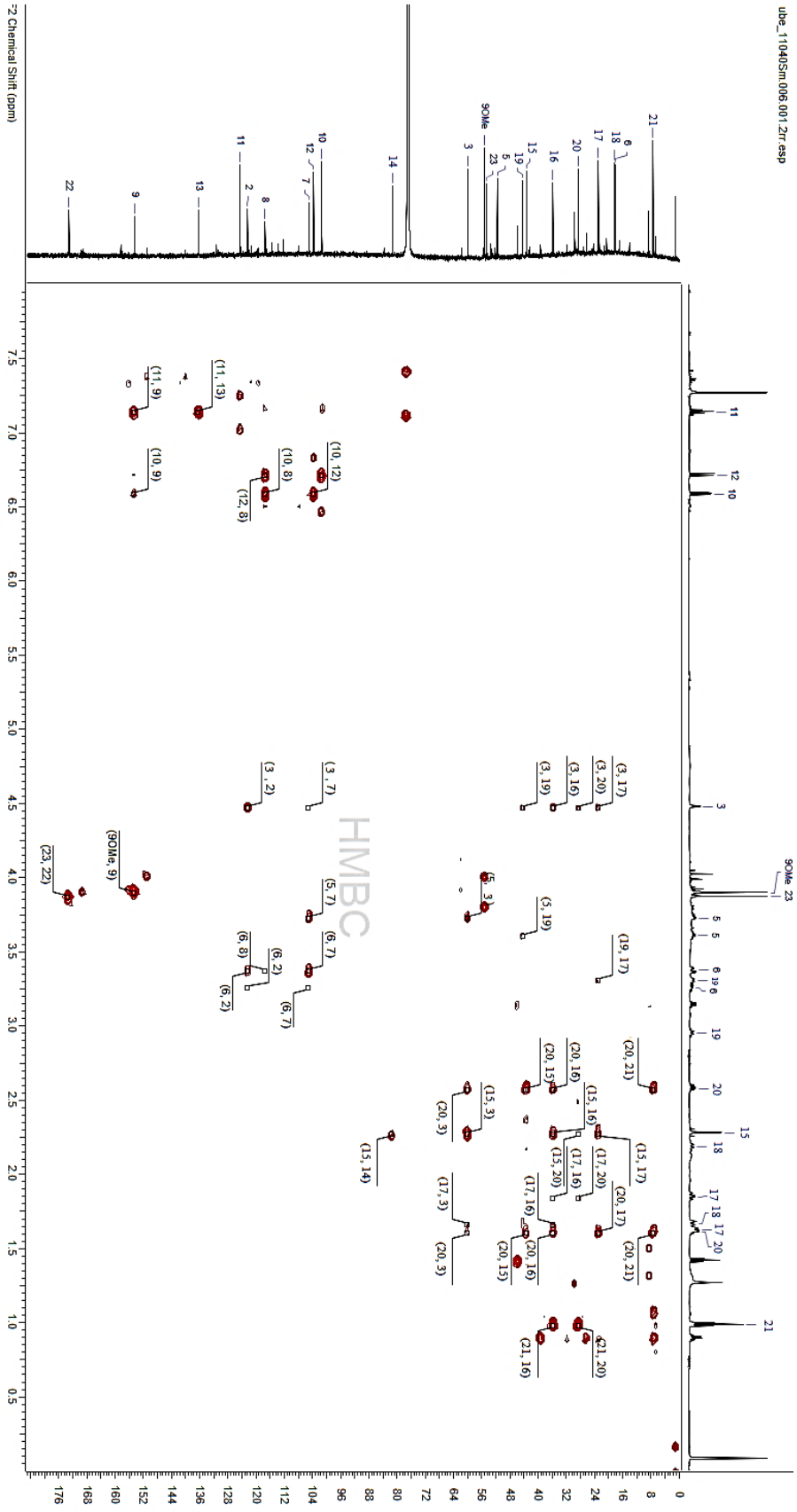
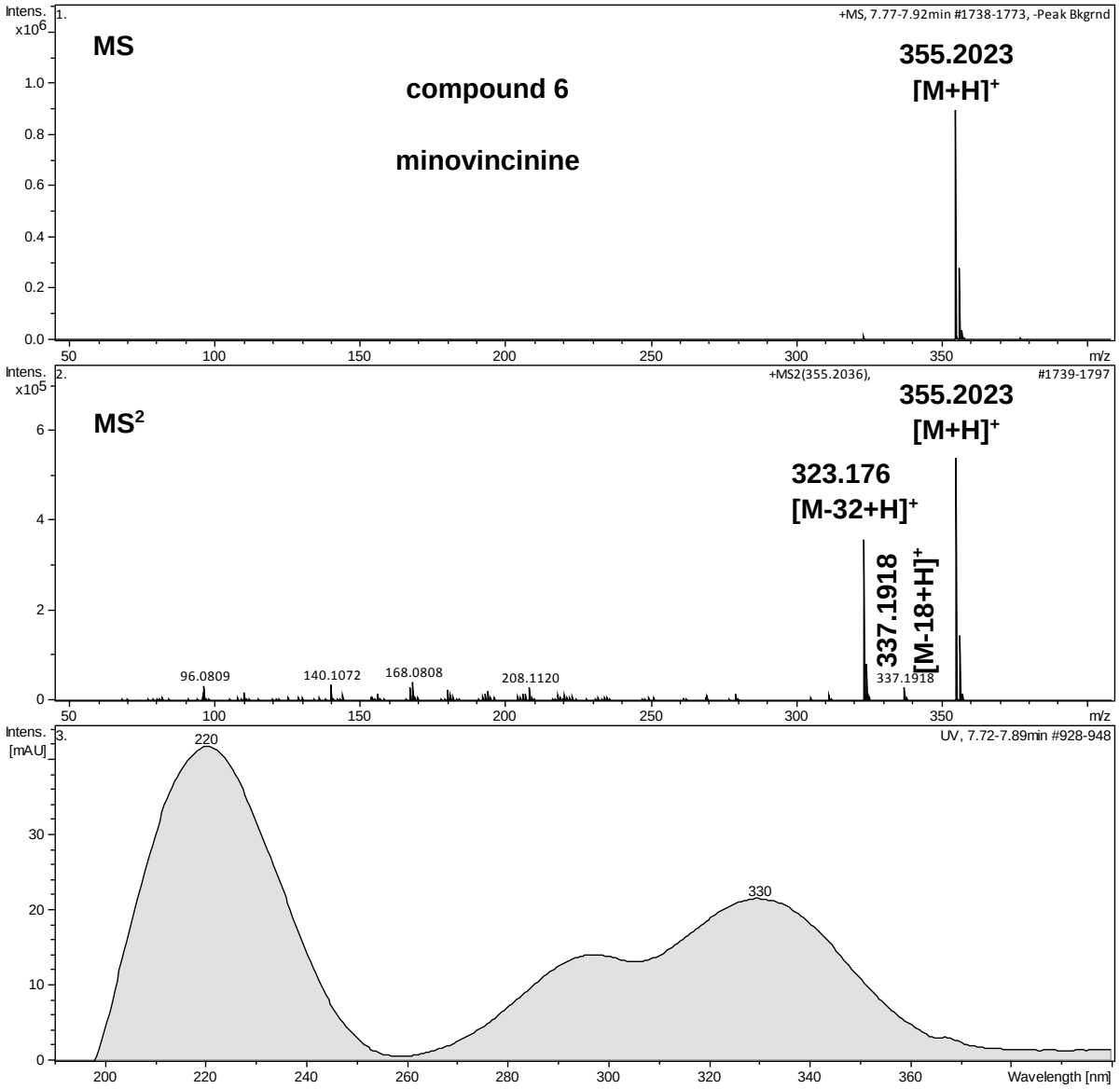


Figure A- 41: ^{1}H-^{13}C-HMBC NMR spectrum (700 MHz, $CHCl_3$-*d*) of 9-methoxyvincamine (**5**) xTFA.



Spectrum Data

Meas. m/z	#	Sum Formula	Ion Formula	Adduct	m/z	err [ppm]	mSigma	# mSigma	Score
355.2023	1	$C_{21}H_{26}N_2O_3$	$C_{21}H_{27}N_2O_3$	M+H	355.2016	-2.0	10.8	1	100.00

Figure A- 42: UV, MS and MS-MS spectrum of minovincinine (**6**).

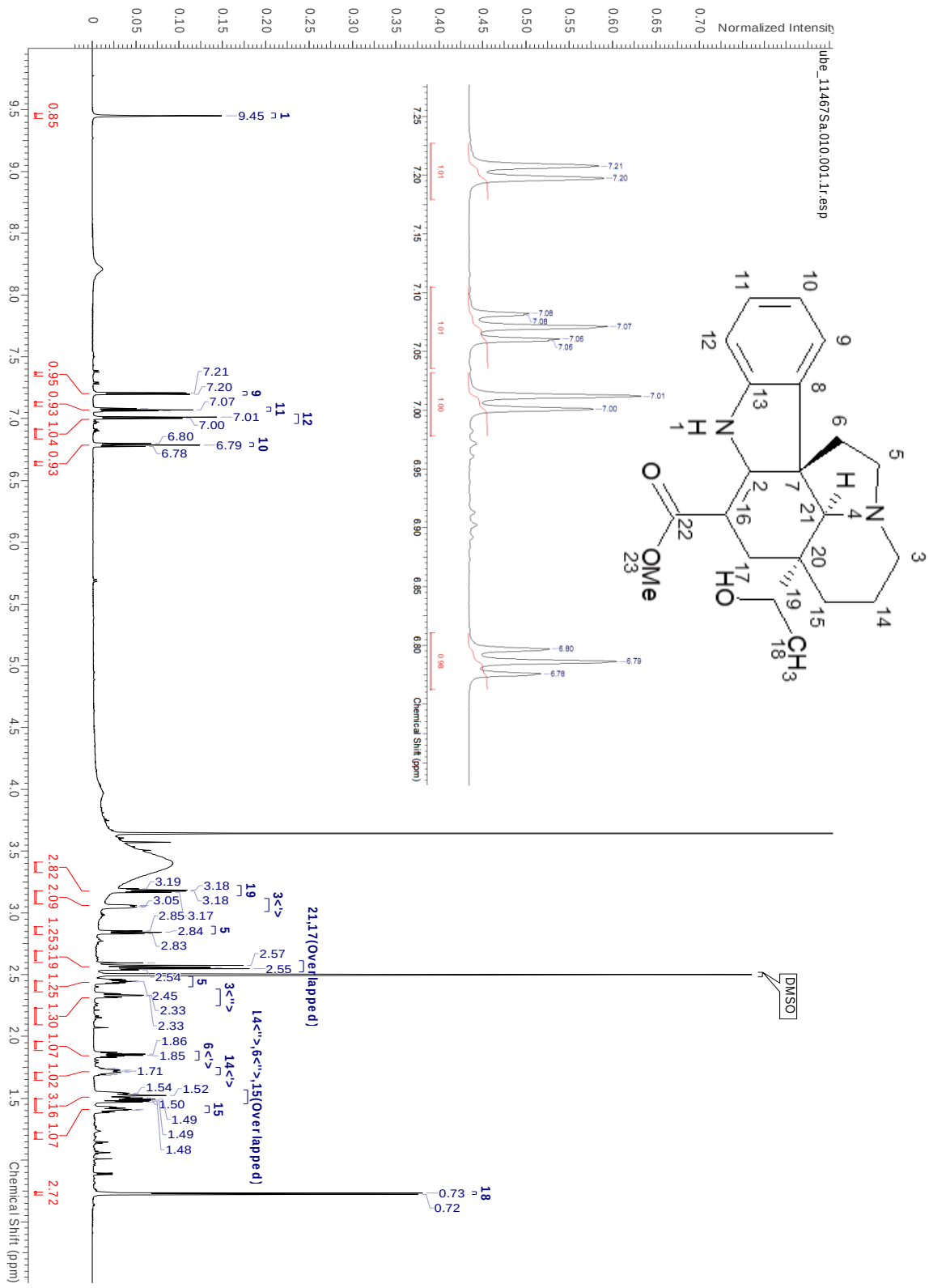


Figure A- 43: ^{1}H- NMR spectrum (700 MHz, DMSO-d_6) of minovincinine (**6**) x formic acid.

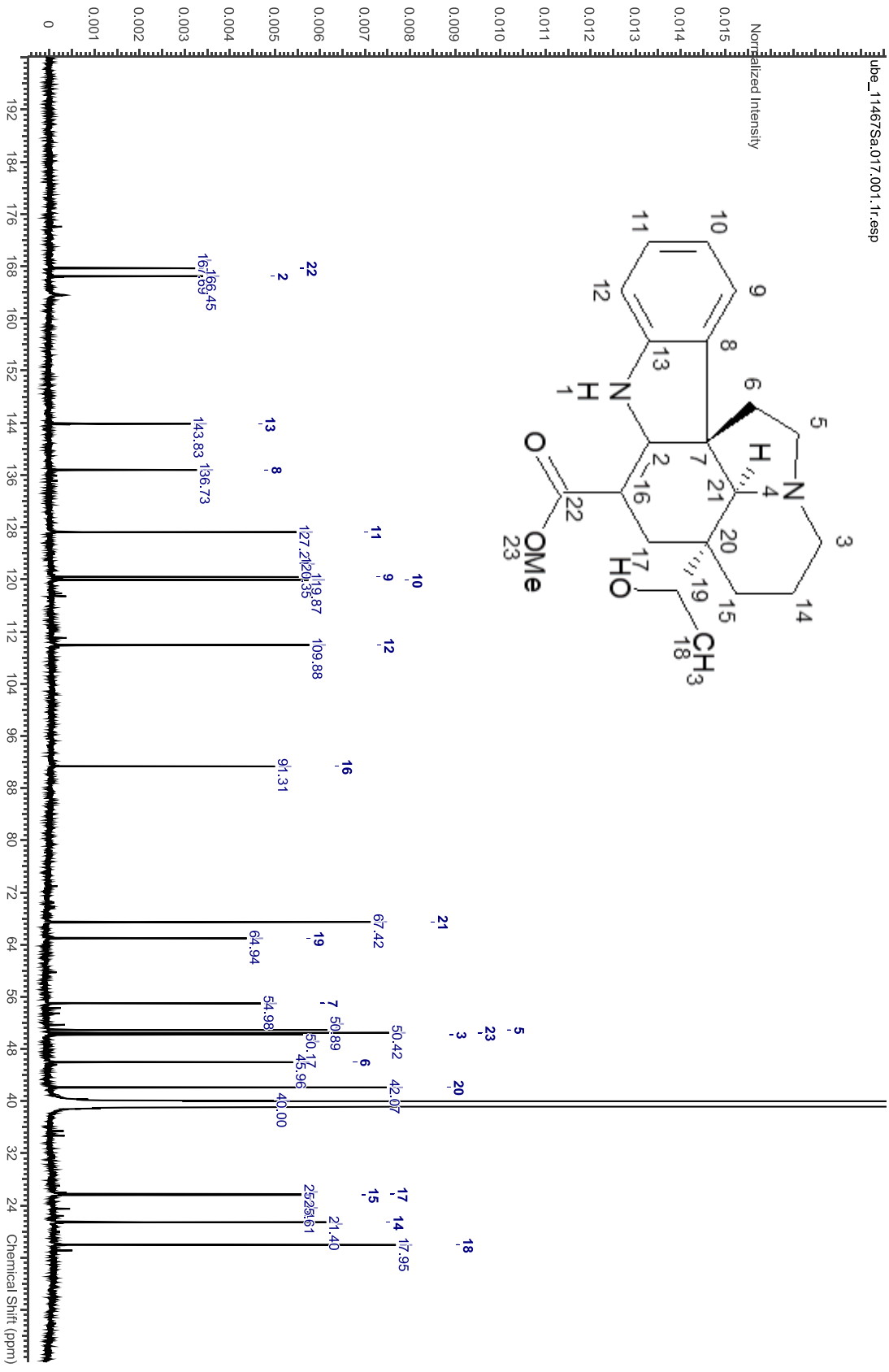


Figure A- 44: ^{1}H-^{13}C-NMR spectrum (175 MHz, DMSO-d_6) of minovincinine (**6**) x formic acid.

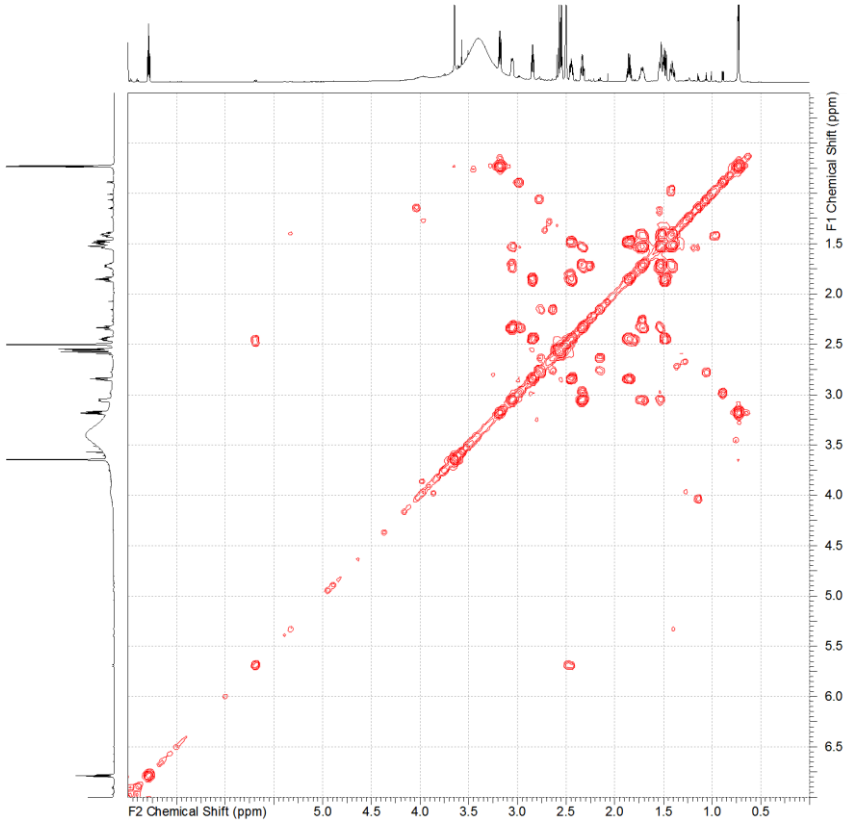


Figure A- 45: ^{1}H-^{1}H-COSYNMR spectrum (700 MHz, DMSO-d_6) of minovincinine (**6**) x formic acid.

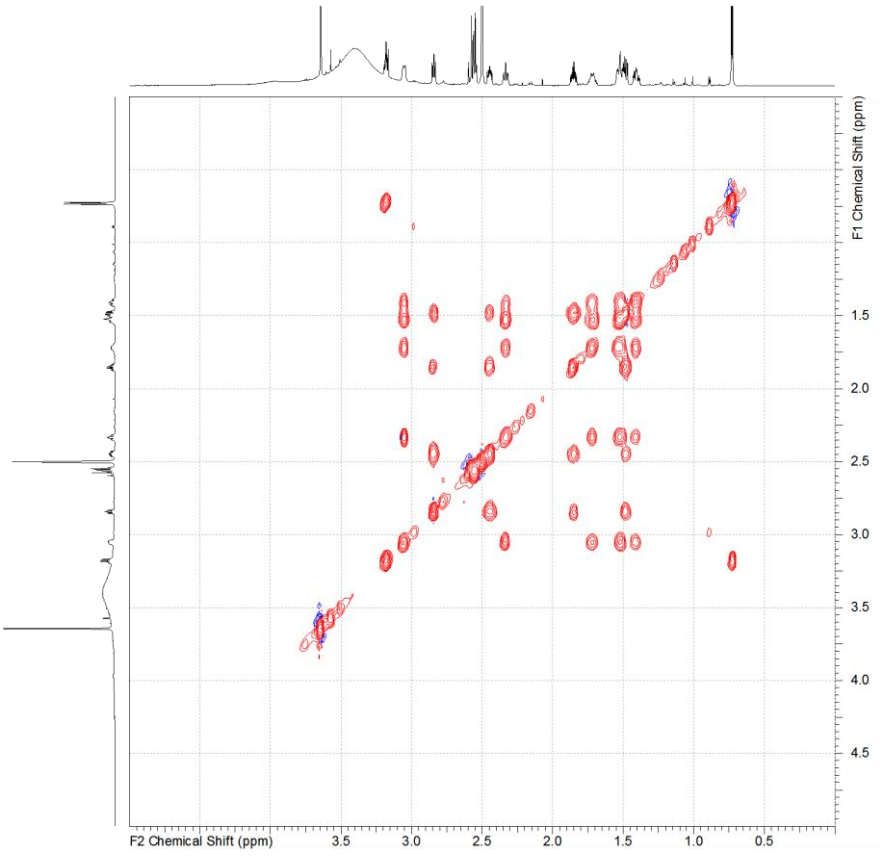


Figure A- 46: ^{1}H-^{1}H-TOCSY-NMR expansion spectrum (700 MHz, DMSO-d_6) of minovincinine (**6**) x formic acid.

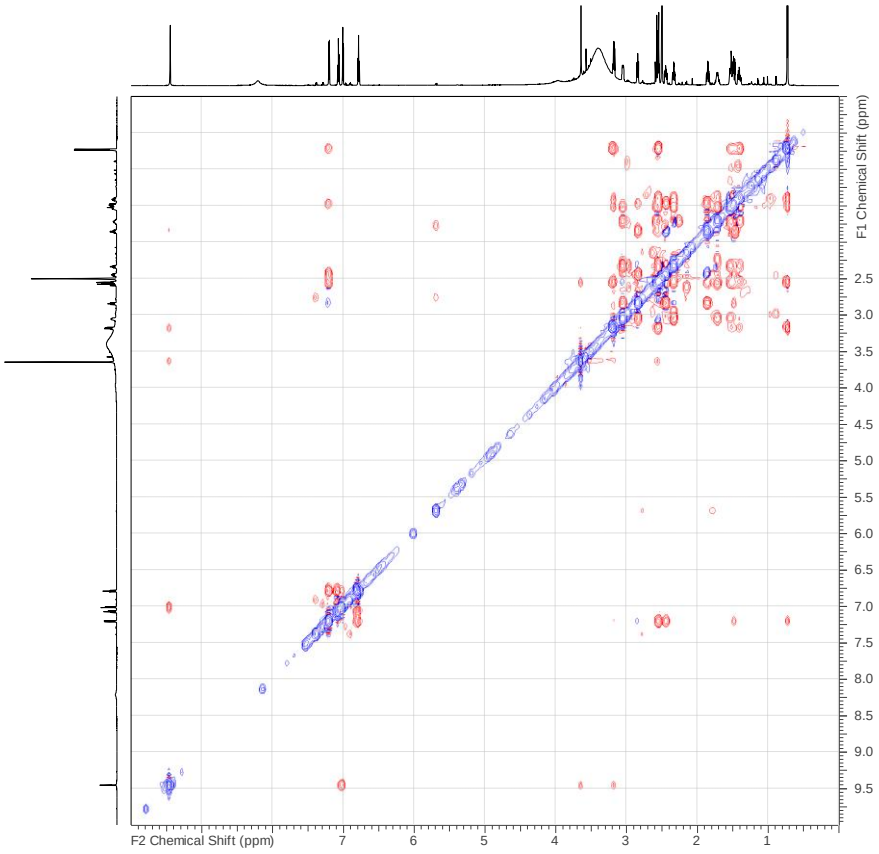


Figure A- 47: ^{1}H-^{1}H-ROESY-NMR pectrum (700 MHz, DMSO-d_6) of minovincinine (**6**) x formic acid.

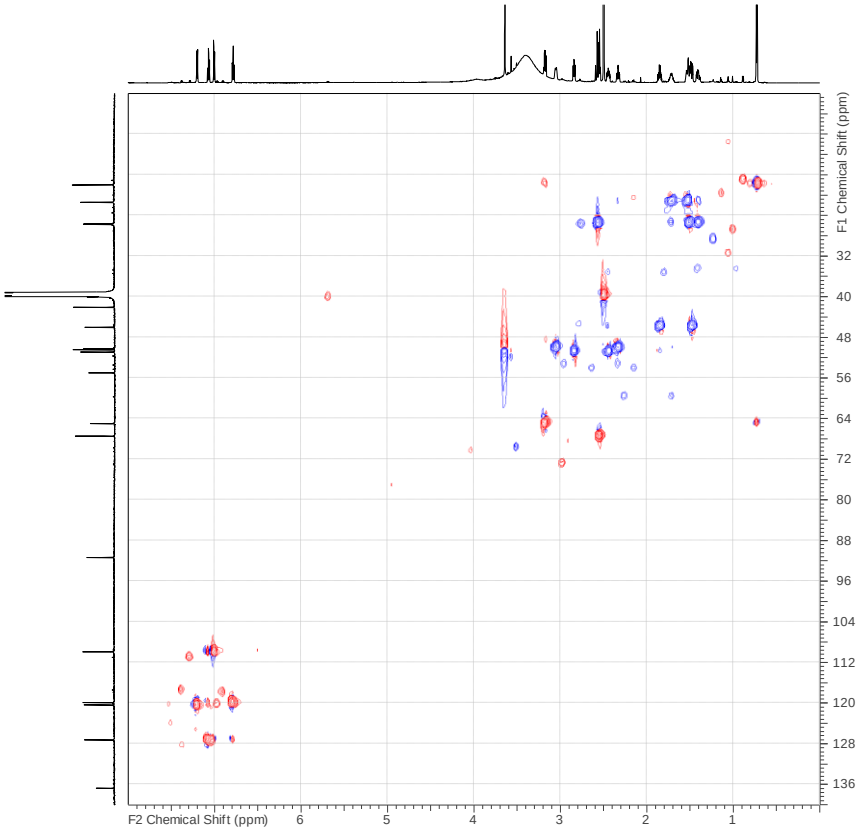


Figure A- 48: ^{1}H-^{13}C-HSQC-DEPT-NMR spectrum (700 MHz, DMSO-d_6) of minovincinine (**6**) x formic acid.

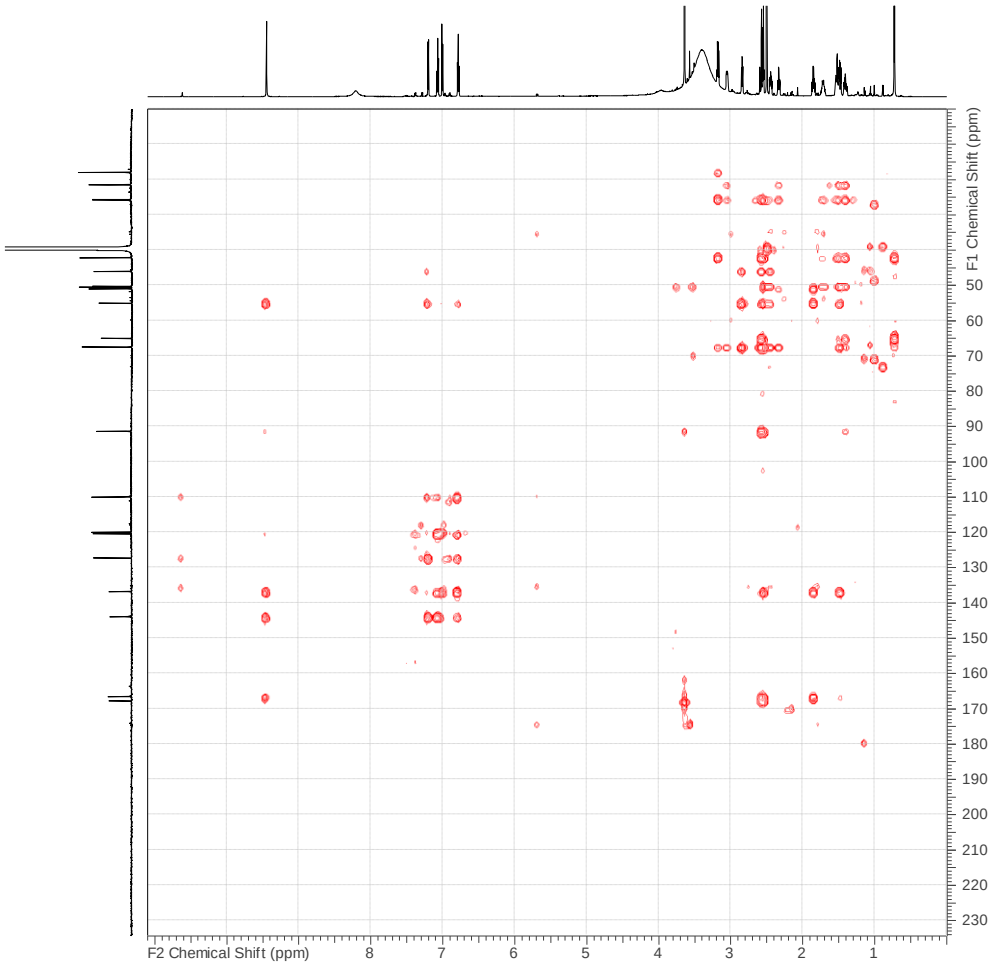
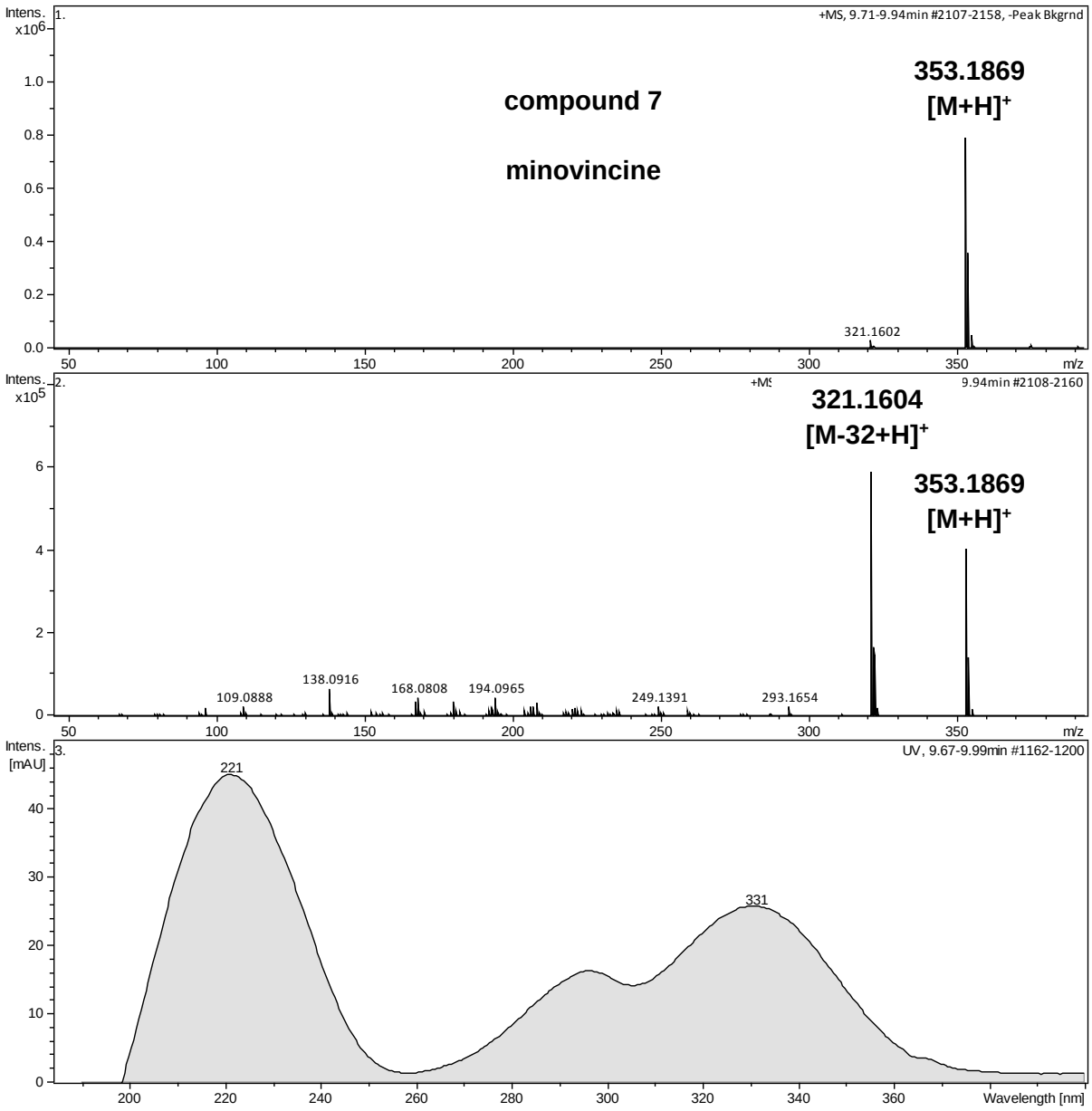


Figure A- 49: ^{1}H-^{13}C-HMBC-NMR spectrum (700 MHz, DMSO-d_6) of minovincinine (**6**) x formic acid.



Spectrum Data

Meas. m/z	#	Sum Formula	Ion Formula	Adduct	m/z	err [ppm]	mSigma	# mSigma	Score
353.1869	1	$C_{21}H_{25}N_2O_3$	$C_{21}H_{25}N_2O_3$	I+H	353.1860	-2.5	13.8	1	100.00

Figure A- 50: UV, MS and MS-MS spectrum of minovincine (**7**)**.**

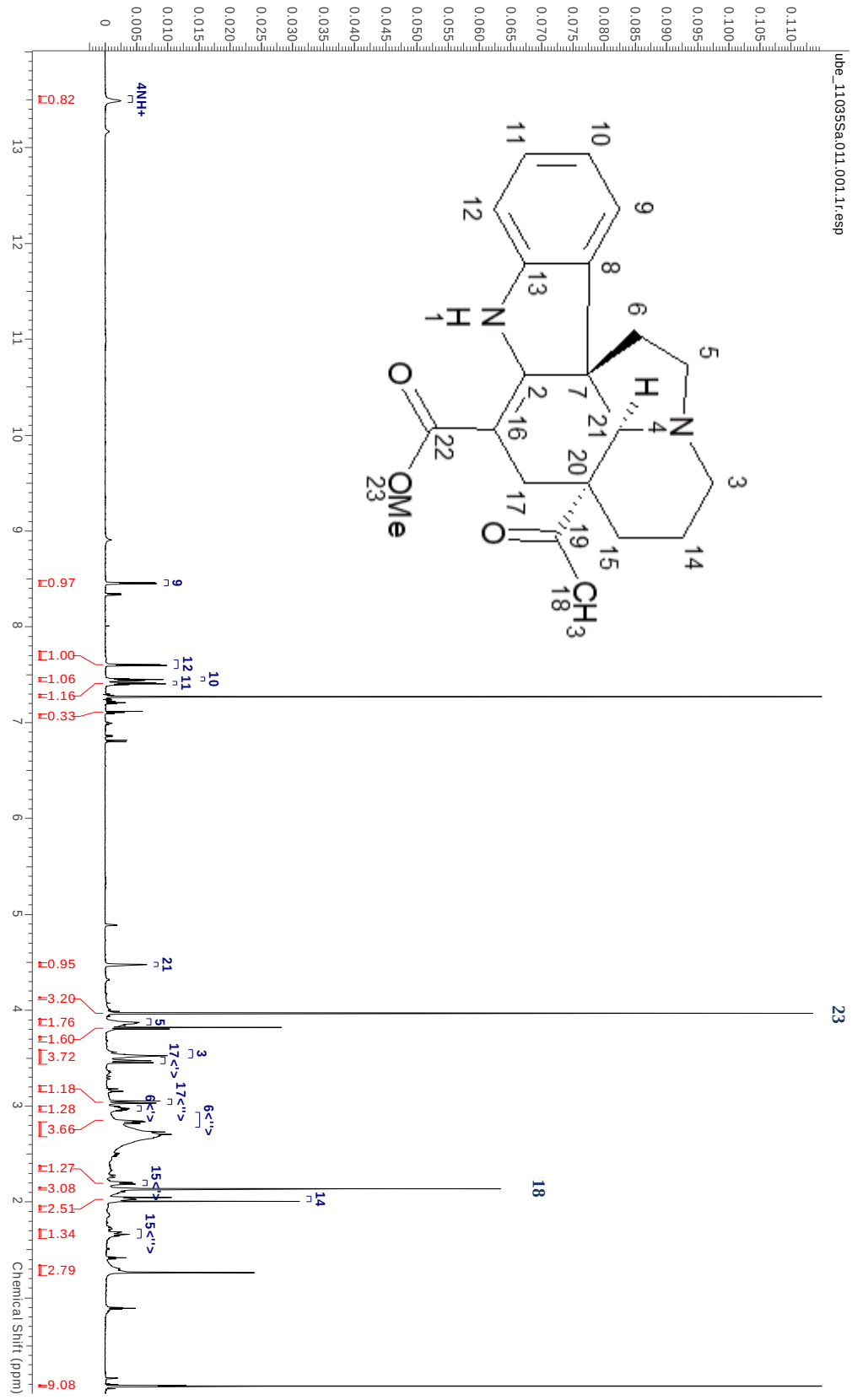


Figure A- 51: ^{1}H NMR spectrum (700 MHz, $CHCl_3$-*d*) of minovincine (**7**) x formic acid.

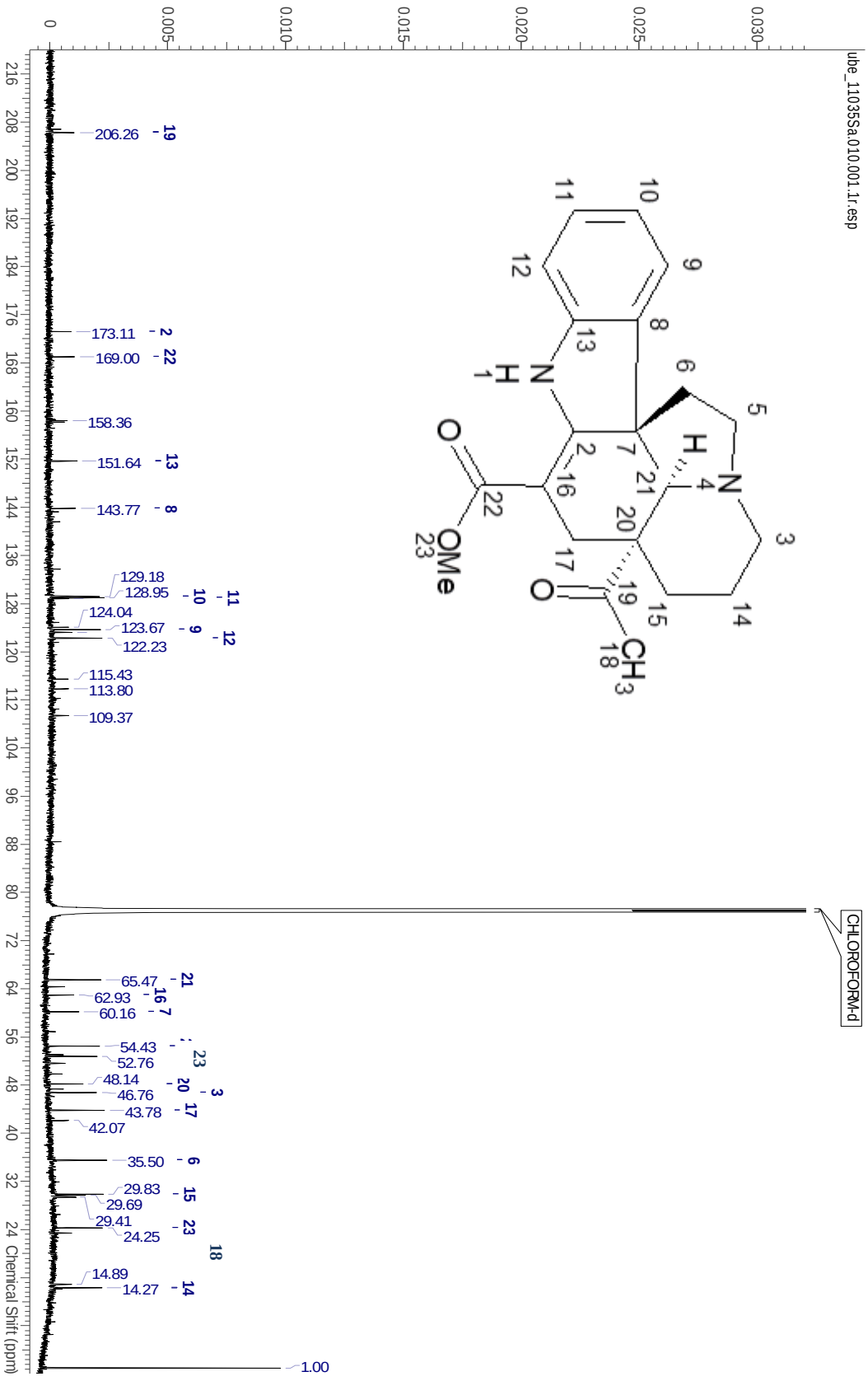


Figure A- 52: . ^{13}C NMR spectrum (175 MHz, $CHCl_3$-*d*) of minovincine (**7**) x formic acid.

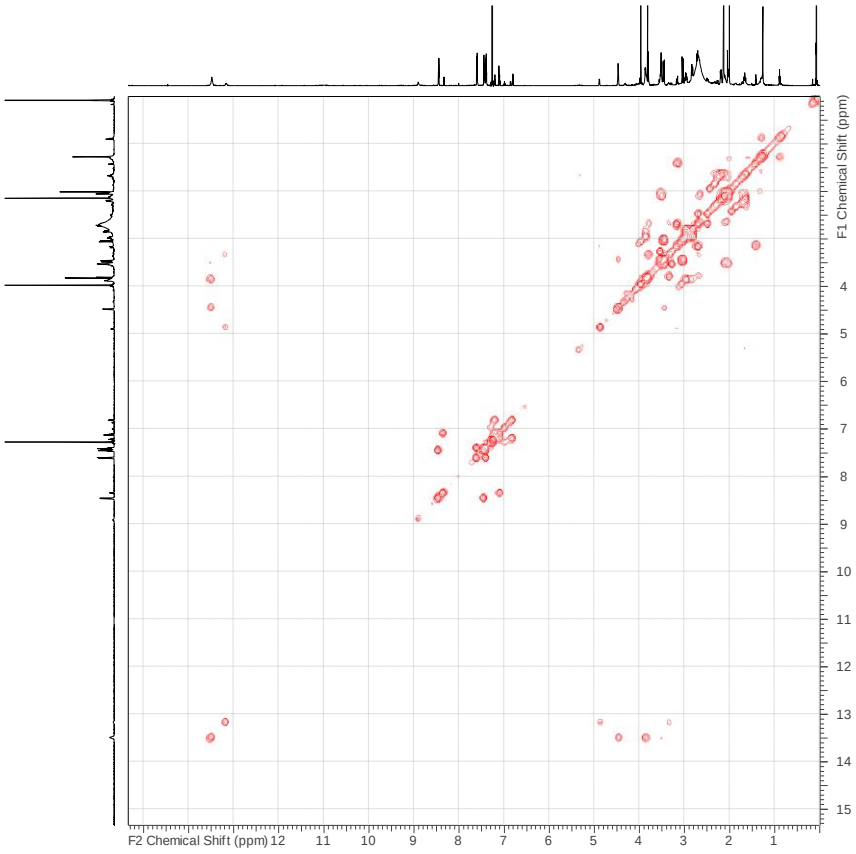


Figure A- 53: ^{1}H-^{1}HCOSY NMR spectrum (700 MHz, $CHCl_3$-*d*) of minovincine (**7**) x formic acid.

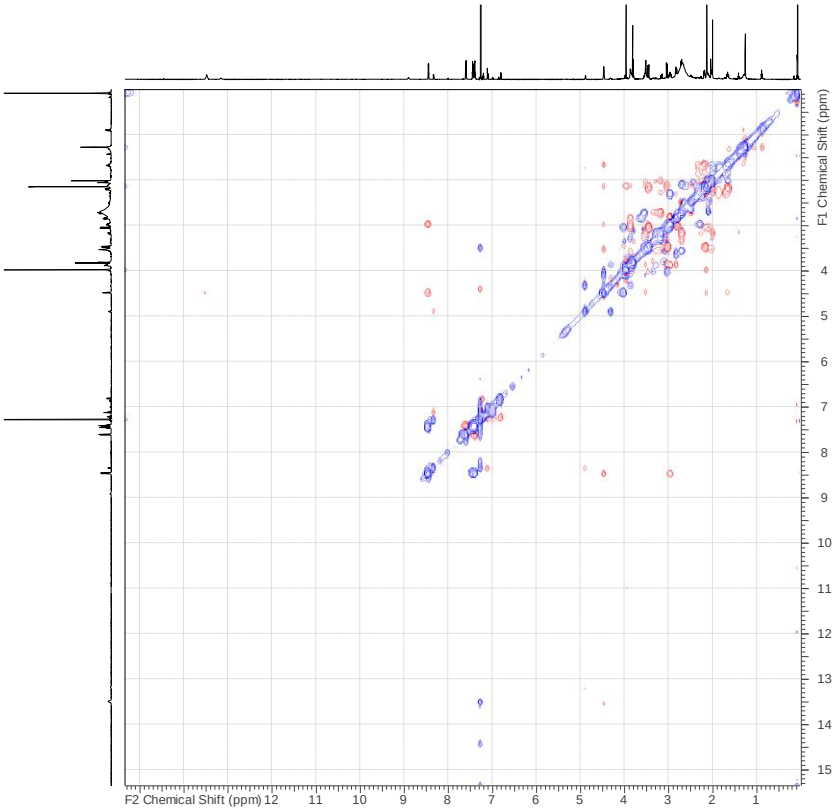


Figure A- 54: ^{1}H-^{1}H-ROESY NMR spectrum (700 MHz, $CHCl_3$-*d*) of minovincine (**7**) x formic acid.

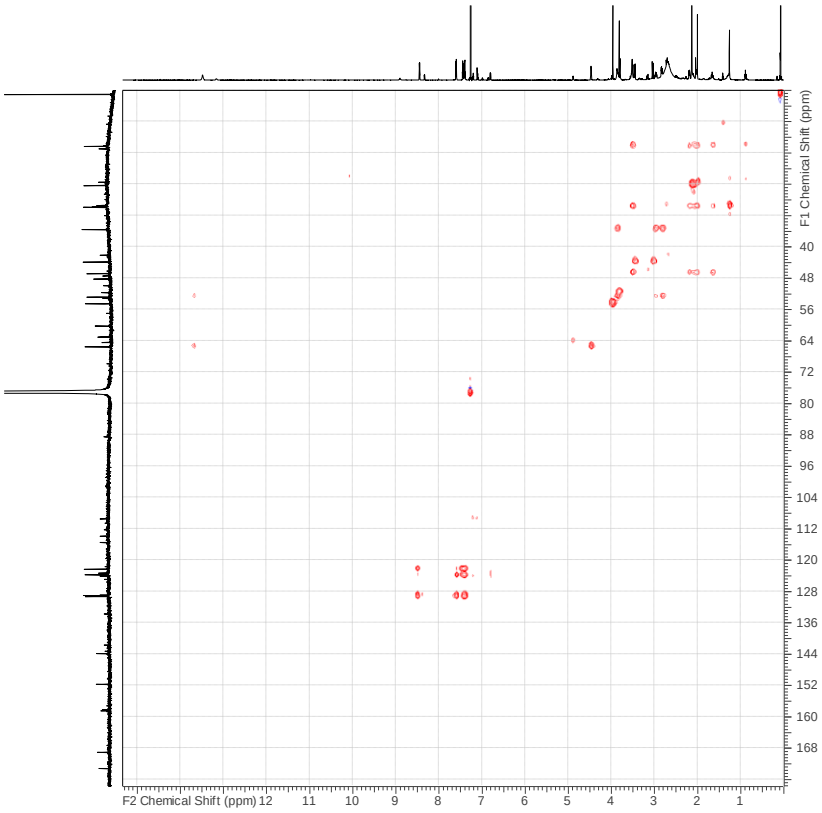


Figure A- 55: ^{1}H-^{13}C-HSQC NMR spectrum (700 MHz, $CHCl_3$-*d*) of minovincine (**7**) x formic acid.

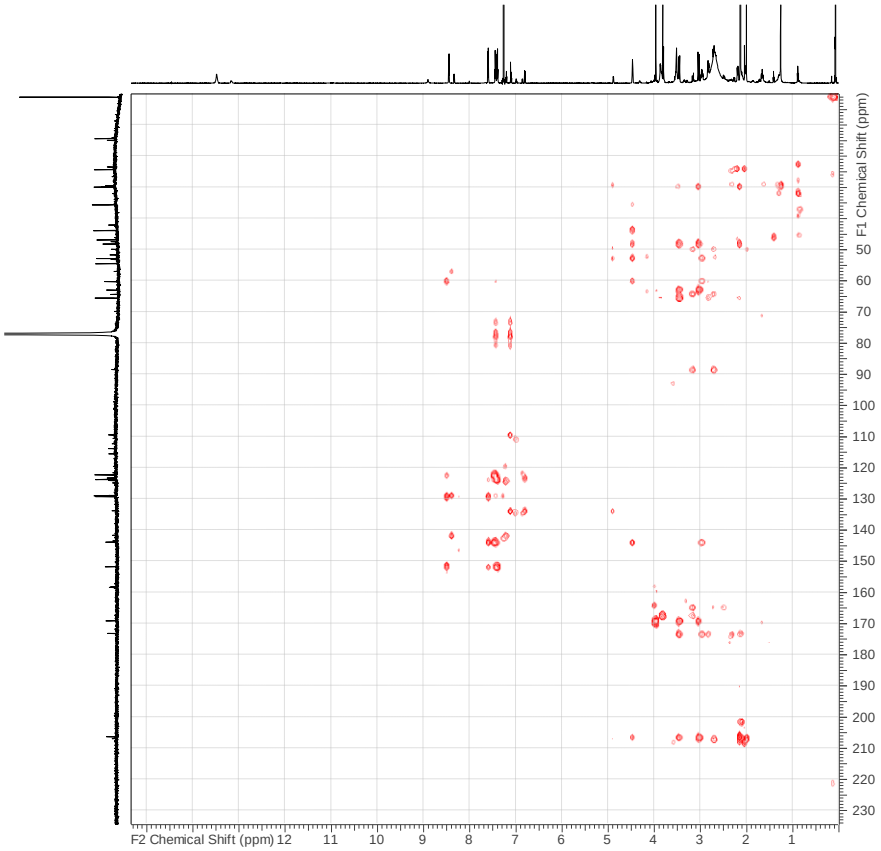
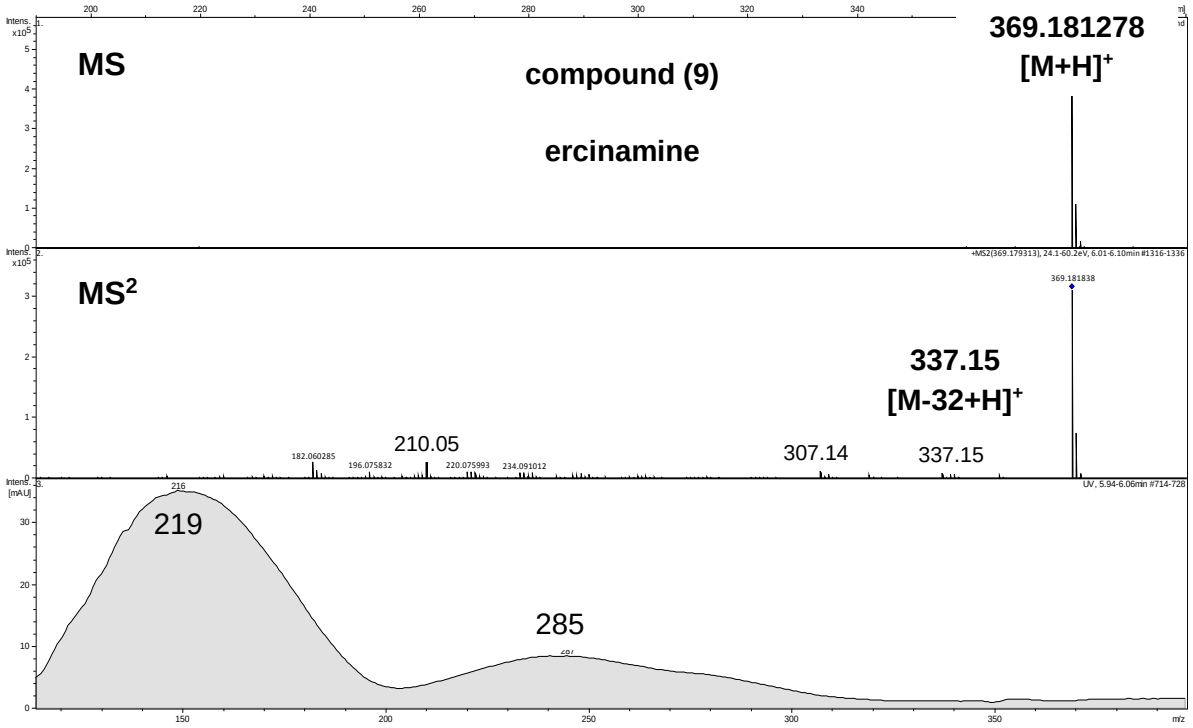


Figure A- 56: ^{1}H-^{13}C-HMBC NMR spectrum (700 MHz, $CHCl_3$-*d*) of minovincine (**7**) x formic acid.



Spectrum Data

Meas. m/z	#	Sum Formula	Ion Formula	Adduct	m/z	err [ppm]	mSigma	# mSigma	Score
369.181871	1	$C_{21}H_{24}N_2O_4$	$C_{21}H_{25}N_2O_4$	M+H	369.180884	-2.674	24.5	1	100.00

Figure A- 57: UV, MS and MS-MS spectrum of ercinamine (**9**).

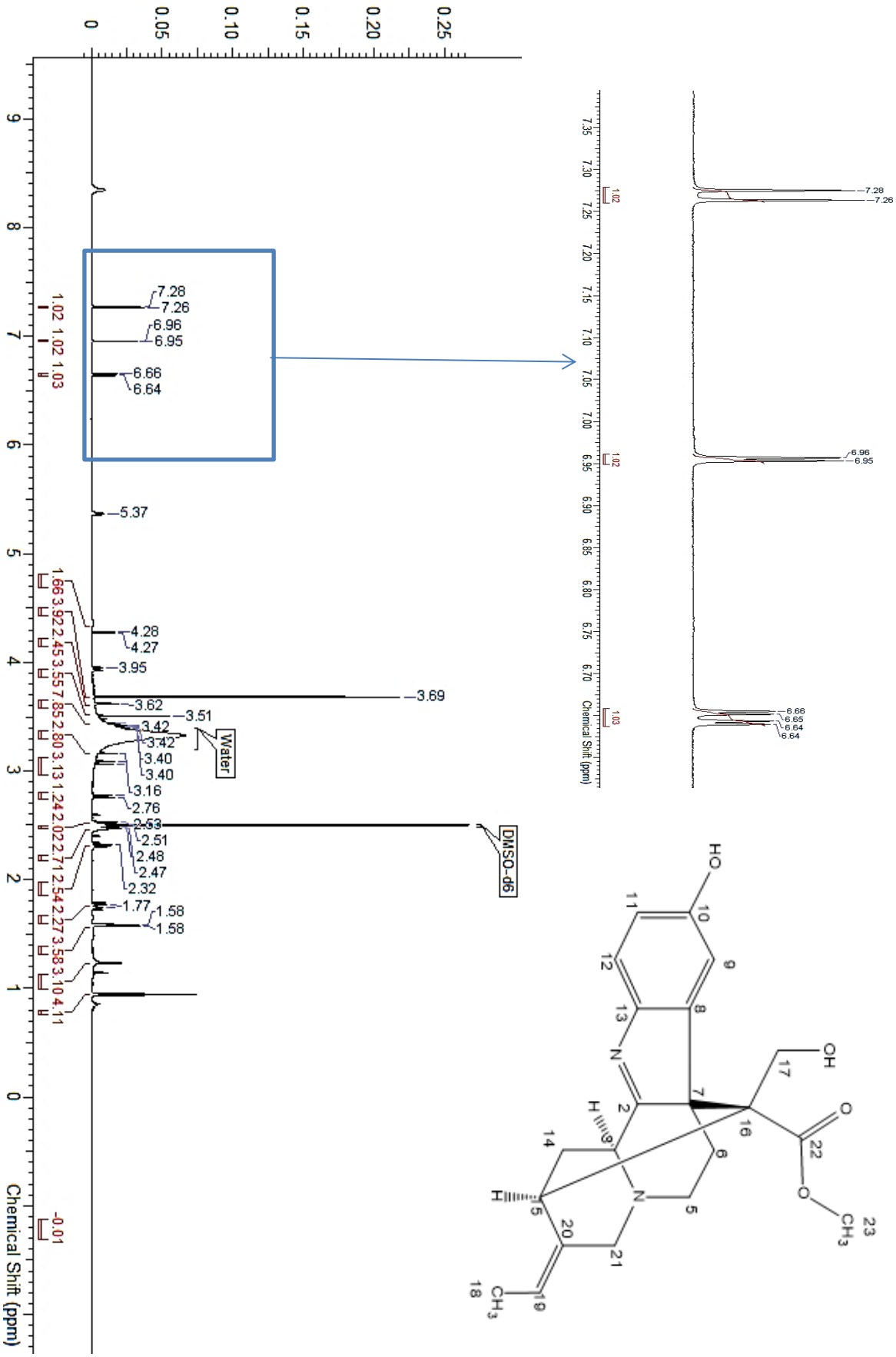


Figure A- 58: ^{1}H- NMR spectrum (700 MHz, DMSO-d_6) of ercinamine (**9**) x formic acid.

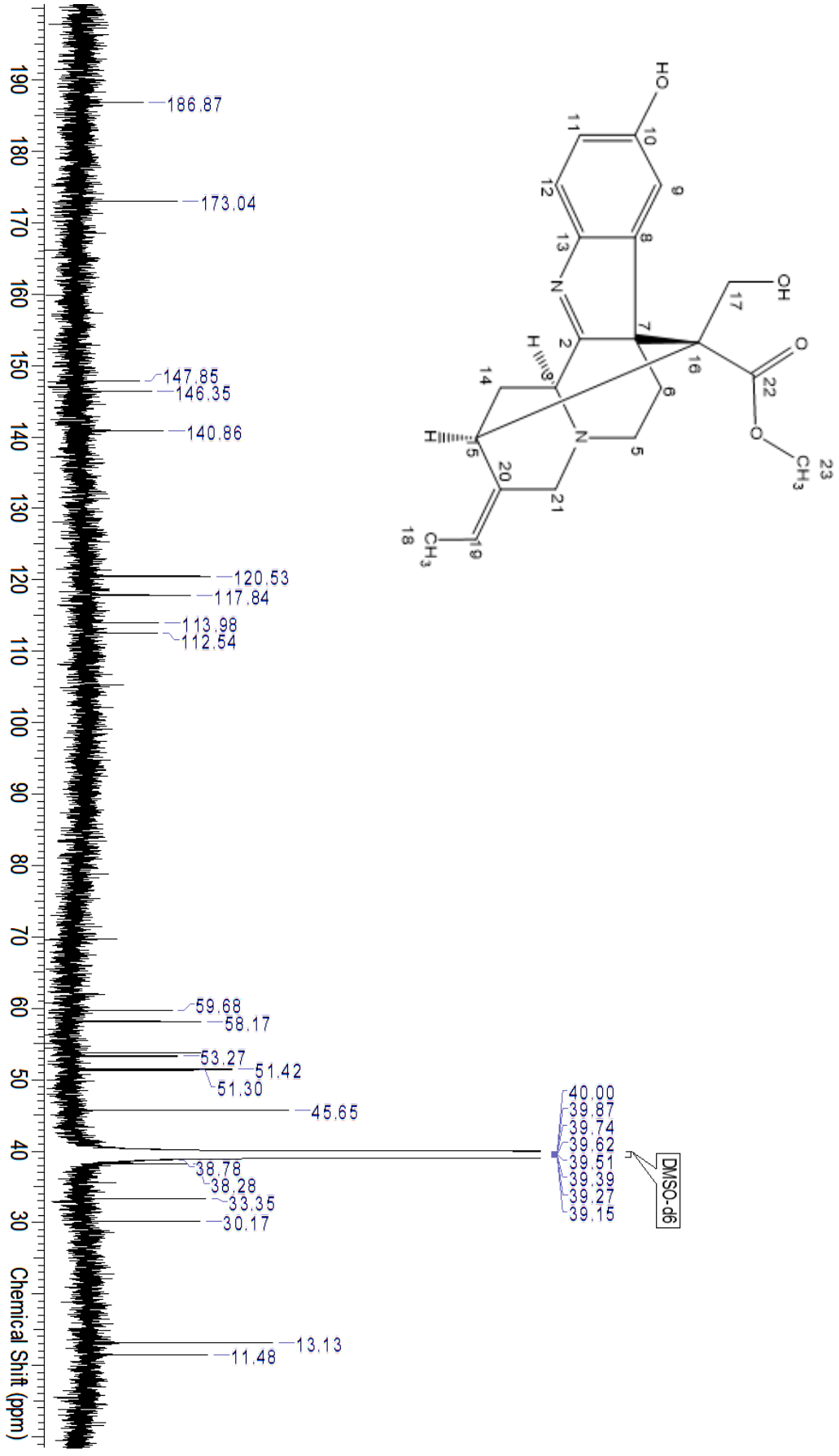


Figure A- 59: ^{13}C- NMR spectrum (175 MHz, DMSO-d_6) of Ercinamine (**9**) x formic acid.

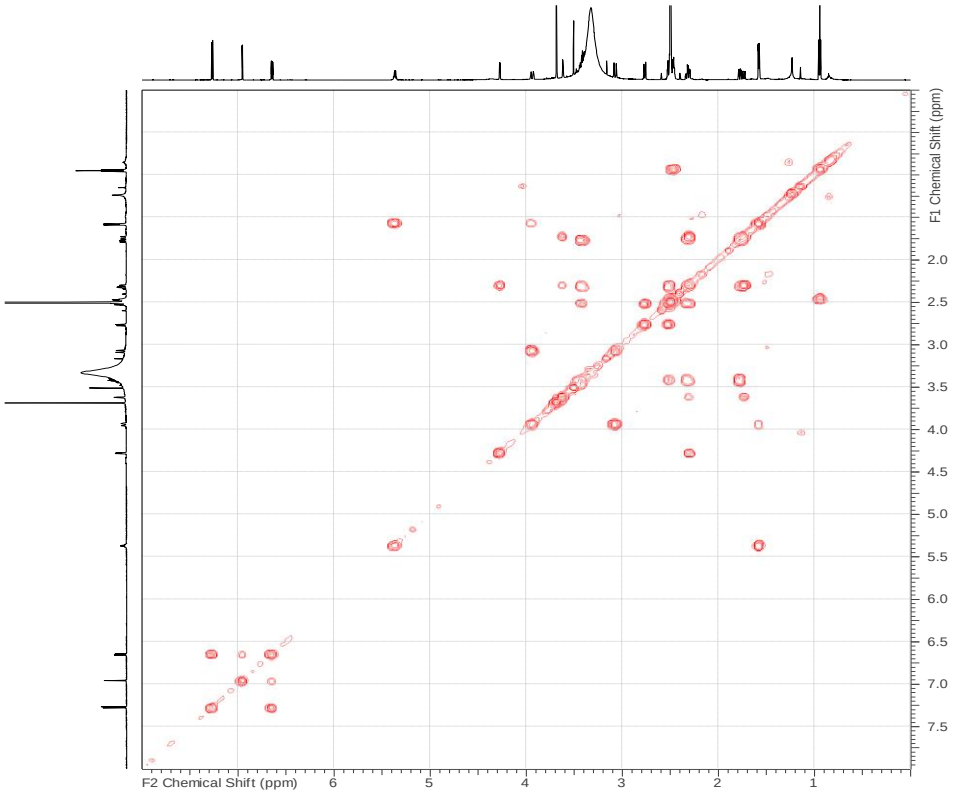


Figure A- 60: ^{1}H-^{1}H- COSY-NMR spectrum (700 MHz, DMSO-d_6) of ercinamine (**9**) x formic acid.

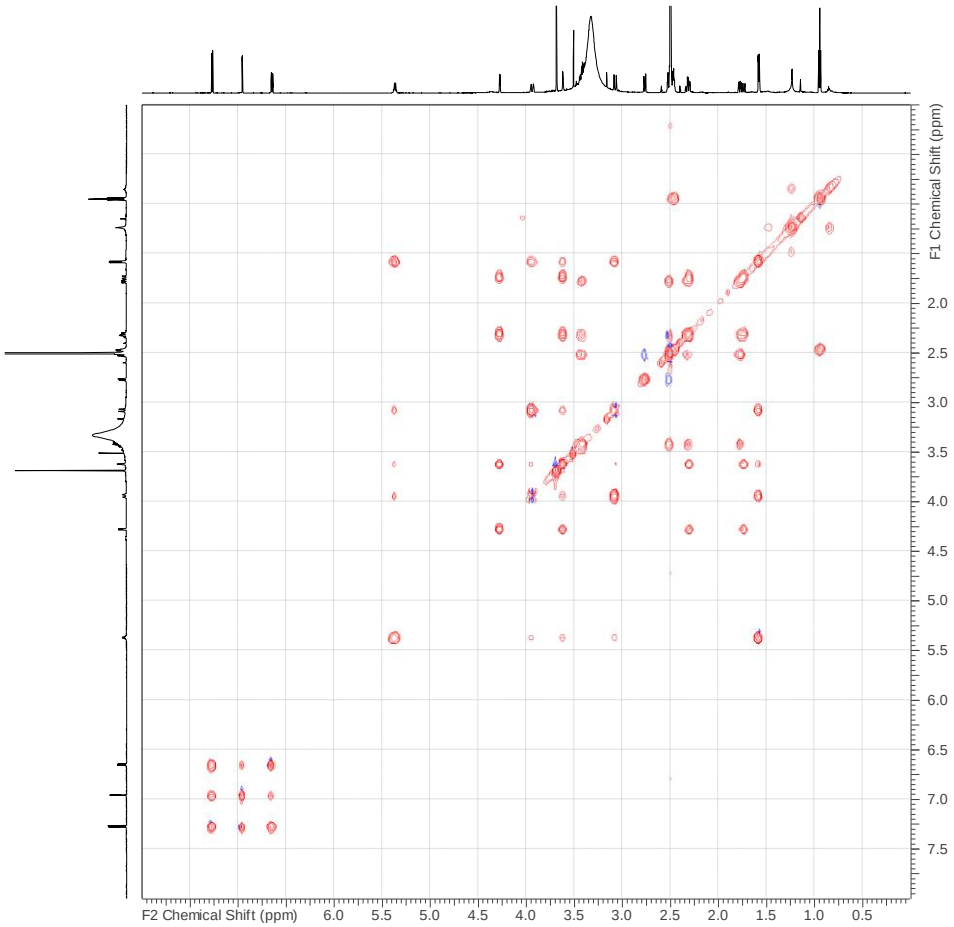


Figure A- 61: ^{1}H-^{1}H- TOCSY-NMR spectrum (700 MHz, DMSO-d_6) of ercinamine (**9**) x formic acid.

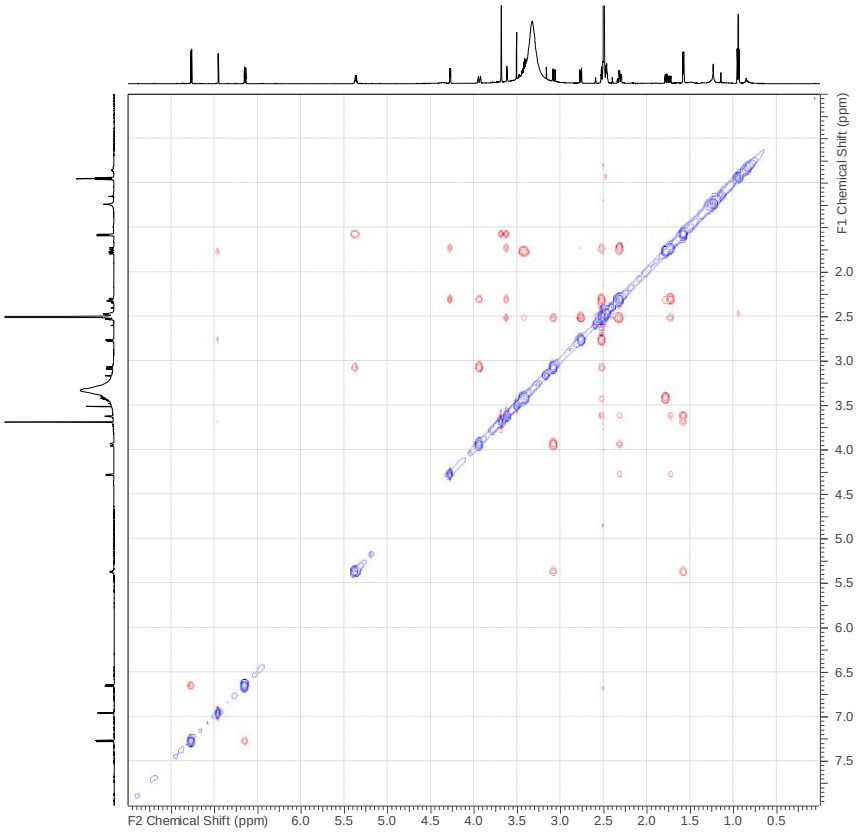


Figure A- 62: ^{1}H-^{1}H- ROESY-NMR spectrum (700 MHz, DMSO-d_6) of ercinamine (**9**) x formic acid.

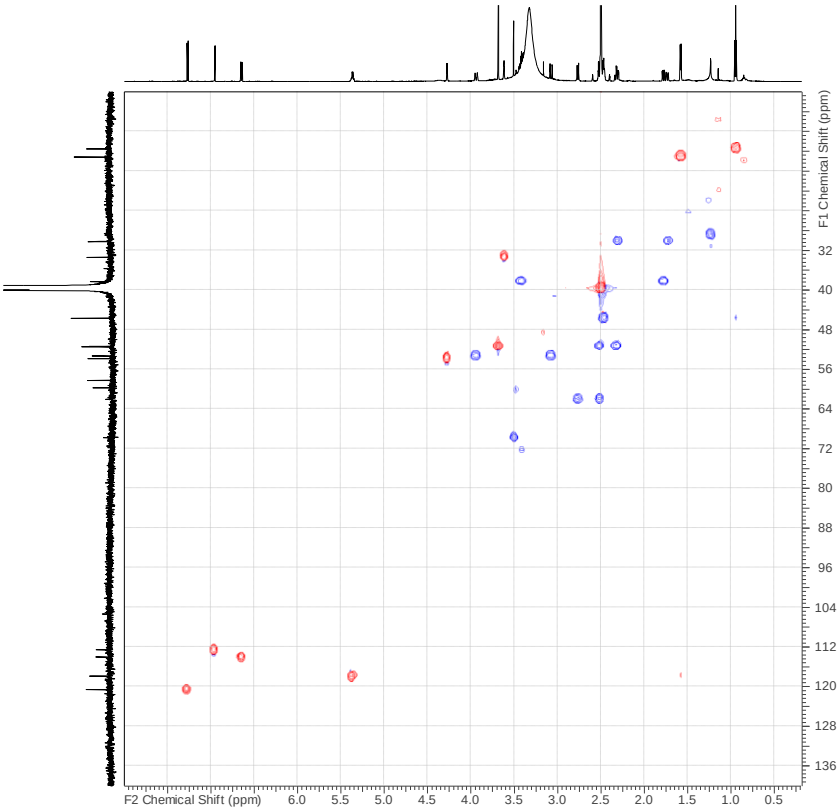


Figure A- 63: ^{1}H-^{13}C- HSQC-DEPT-NMR spectrum (700 MHz, DMSO-d_6) of ercinamine (**9**) x formic acid.

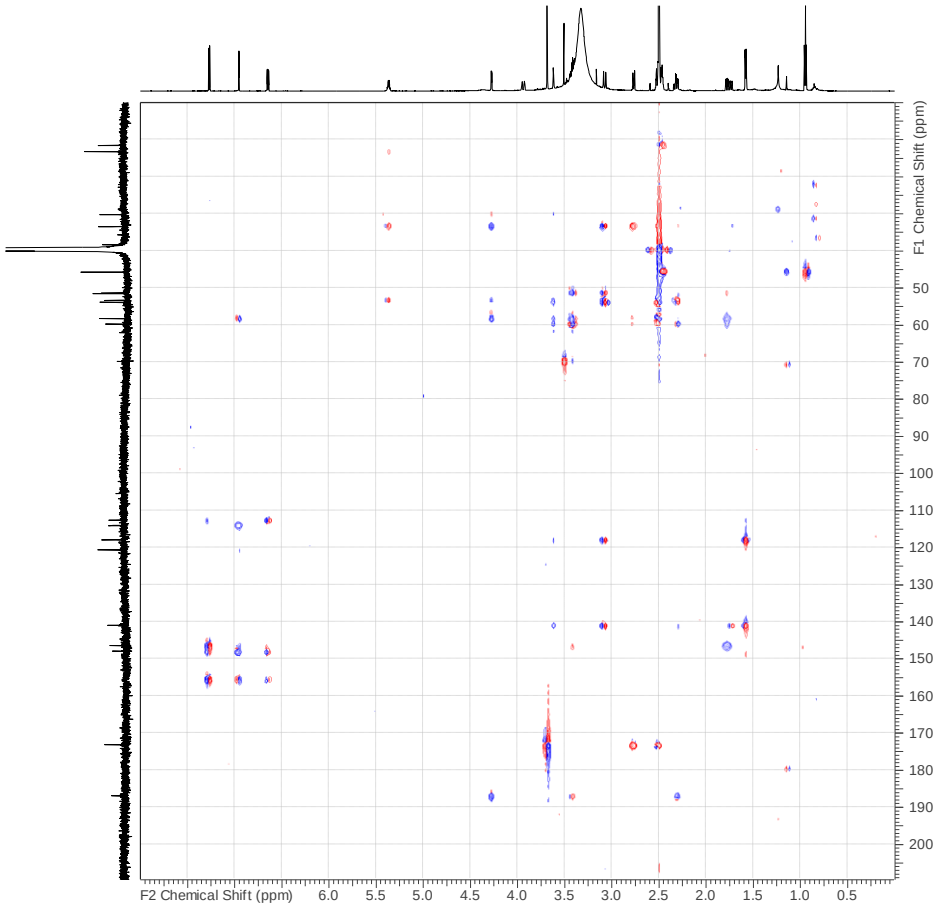


Figure A- 64: ^{1}H-^{13}C- HMBC-NMR spectrum (700 MHz, DMSO-d_6) of ercinamine (**9**)x formic acid.

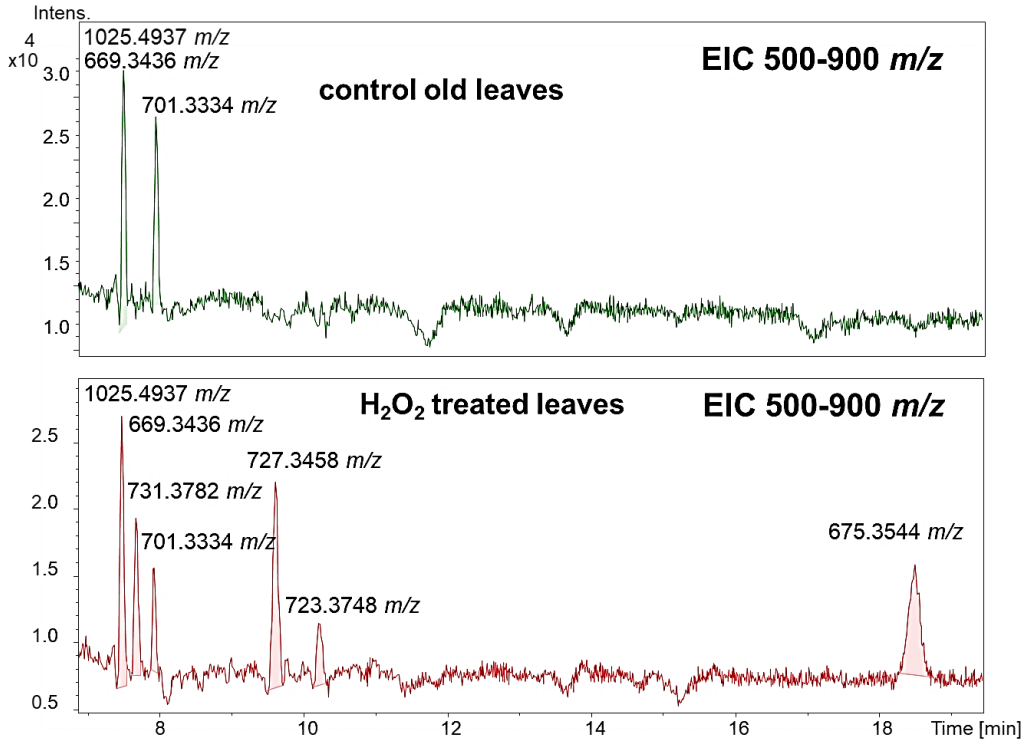


Figure A- 65: The extracted ion chromatograms (EIC 500-900 *m/z*) of control and H_2O_2 treated old leaves. It turned out that several unique dimeric alkaloids are present only on the stressed plants. Unfortunately, the MS-MS fragmentation is not very intense for solid structures deductions.

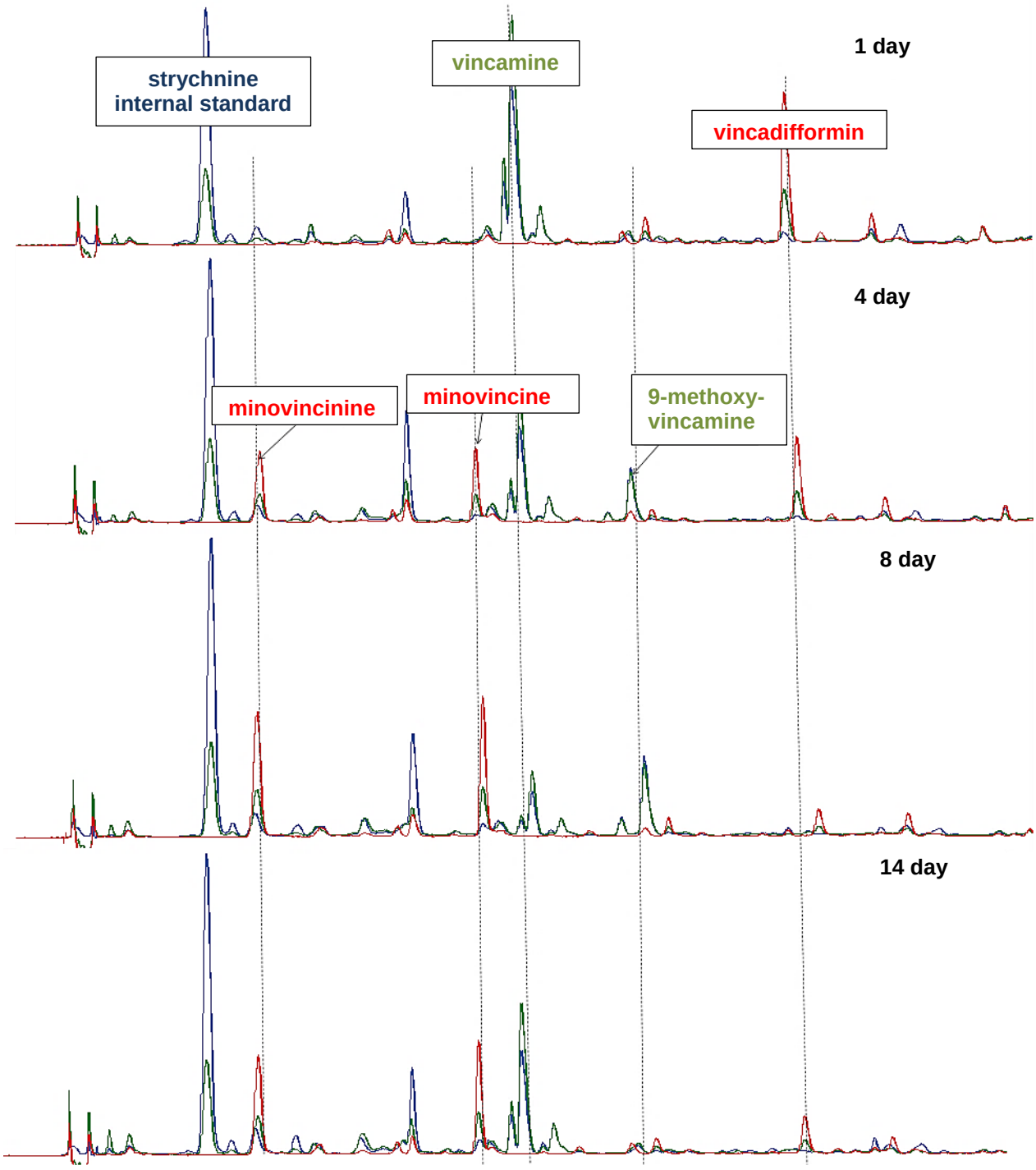


Figure A- 66: The different response of the plants to salicylic acid (SA) during the experiment. In case of SA no significant change in the spectrum of the alkaloids was observed; except for single plant after 4 and 8 which showed a drastic increase in 9-methoxyvincamine, minovincinine and minovincine and decrease in vincamine and vincadifformine. After 14 days, 9-methoxyvincamine was present in very low concentration, whereas minovincinine and minovincine were present in high concentration. Since the reasons for this particular and unique finding are unknown.

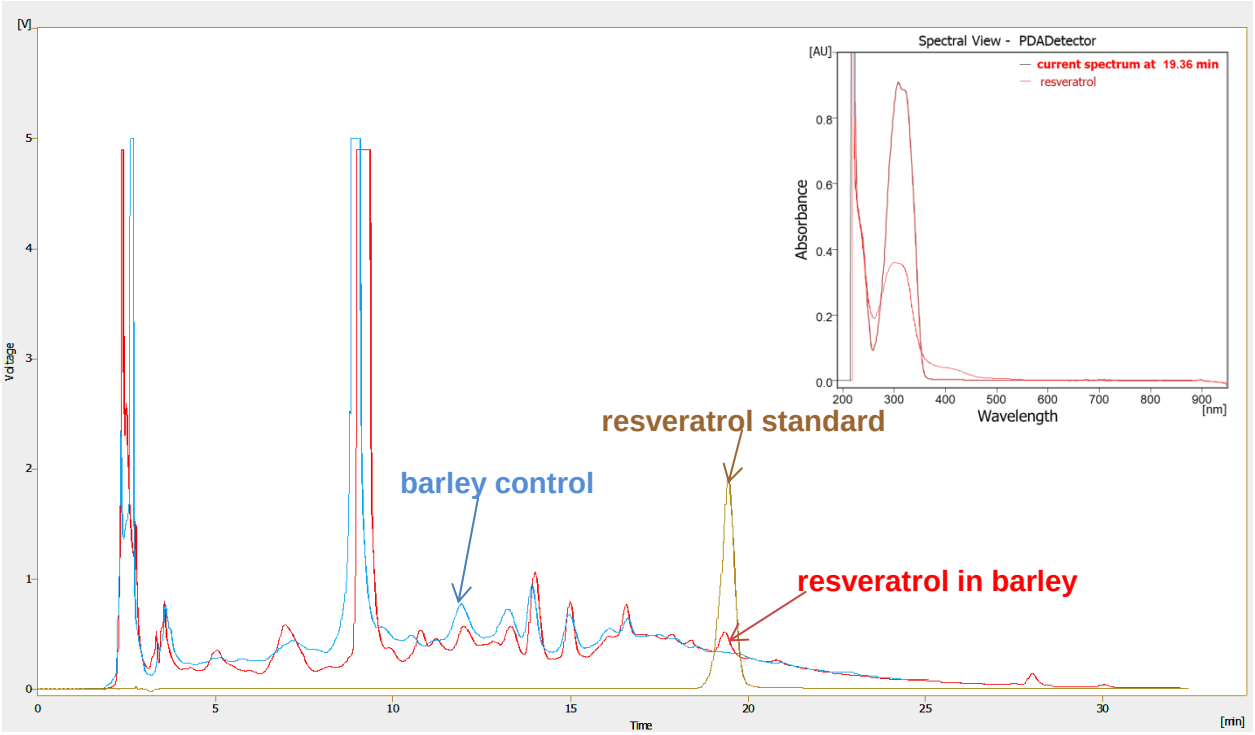


Figure A- 67: Uptake of the resveratrol by the barley plant. Resveratrol was applied to the irrigated water of barley plant with the same concentration used in *Vinca* experiment.

Plant material and application of resveratrol:

Barley seedlings were sown in vermiculite. Two weeks after germination, resveratrol was added during watering as mentioned with *Vinca minor*. To exclude any contamination with the resveratrol-containing substrate (vermiculite), harvesting, was performed by cutting the seedlings at 1–2 cm above the ground after one week.

HPLC analysis of resveratrol:

After grinding the freeze-dried plant material using a ball mill, 0.6g of the powdered sample were extracted with 10 mL methanol and sonicated for 30 min at 50 ºC. After centrifugation (10 min; 10,000 x g). The supernatant was pooled and evaporated using a steam air, facilitated using a water bath at 45ºC. The dry residue was dissolved in methanol. After sonication, the solution was filtered and used for HPLC analysis.

HPLC was performed using a Nucleosil RP-C18 column by applying a binary gradient, starting with 85% A, 15% B. After 10 min the ratio was 76% A, 24% B; subsequently the ratio was changed as follows:30 min: 38% A, 62% B; 33 min: 30% A, 70% B; using A: aqueous acetic acid (1%) and B: acetonitrile. The flow rate was 0.8 mL/min and the injection volume was 50 µL. For detection, a PDA detector was applied (304 nm). Identification was made by comparison to authentic reference resveratrol.

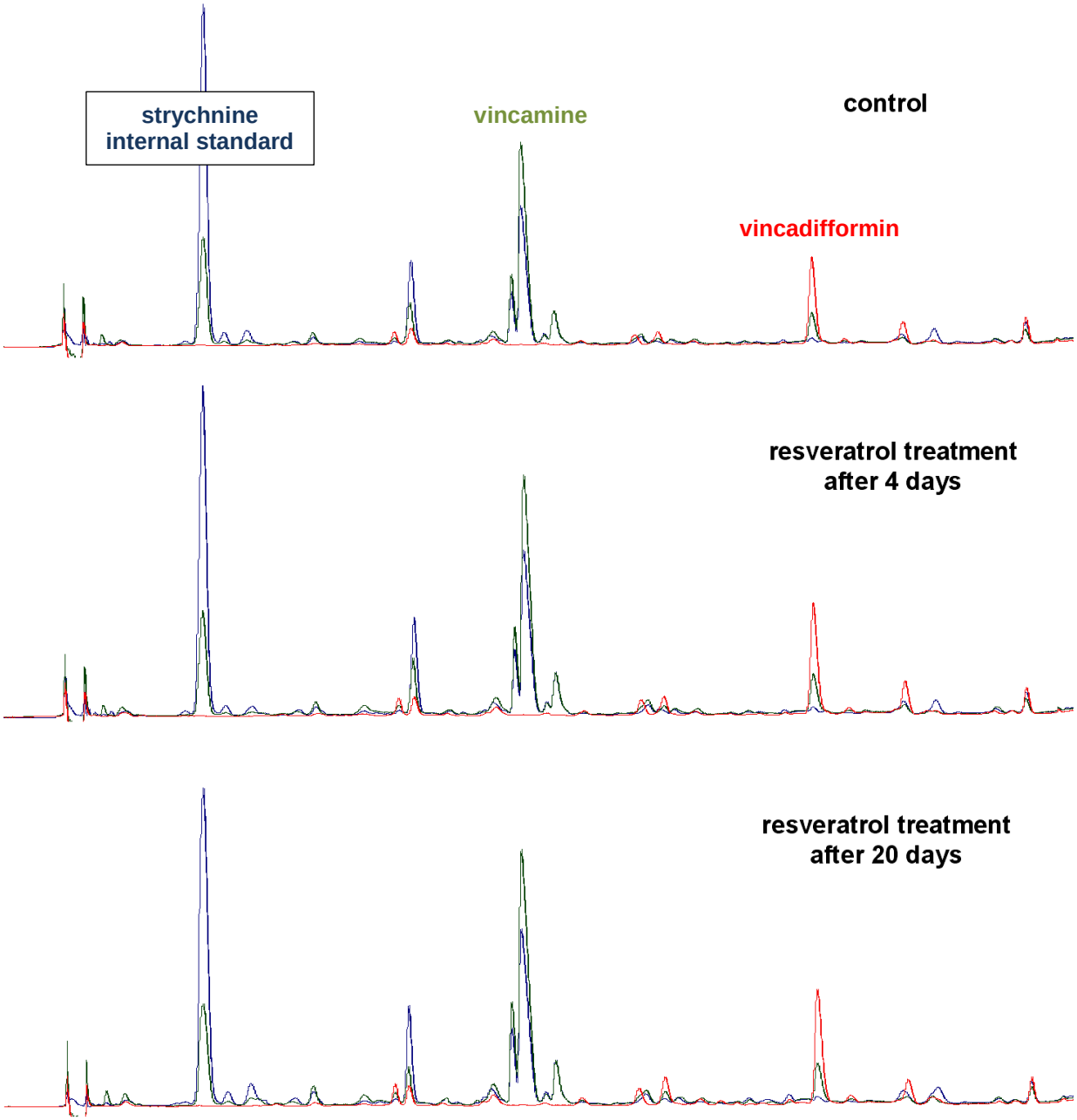


Figure A- 68: The response of the plants to resveratrol during the experiment. In case of resveratrol no significant change in the spectrum of the alkaloids was observed.

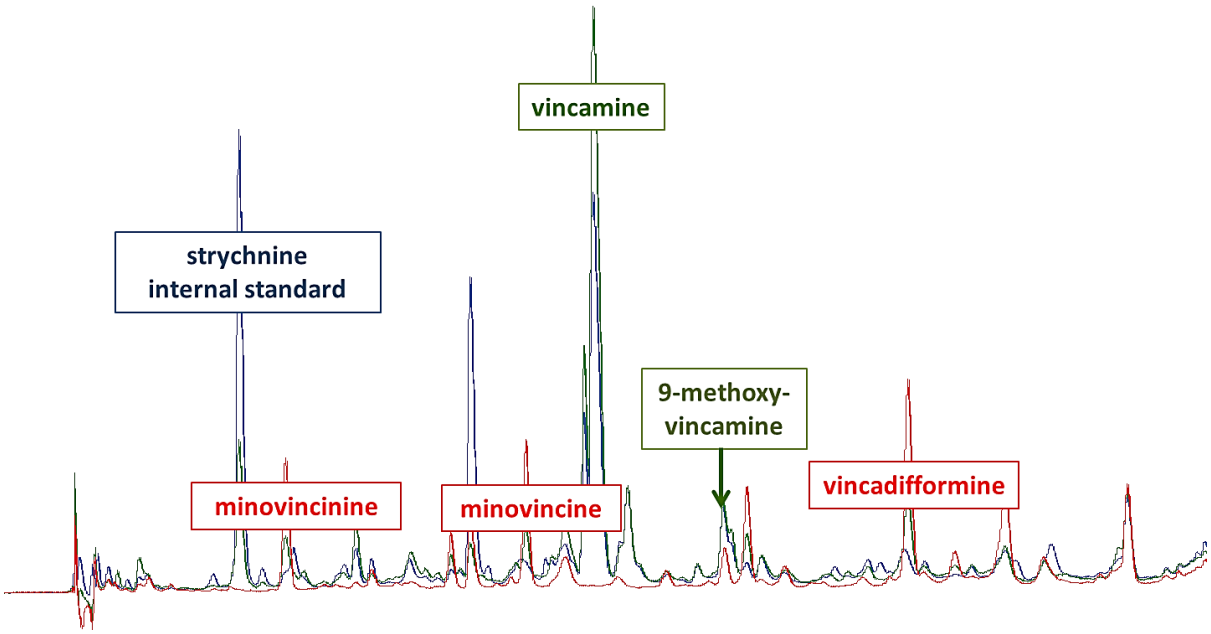


Figure A- 69: Impact of the concomitant application of naproxen and MeJA on the alkaloid spectrum *V. minor* after 3 days.

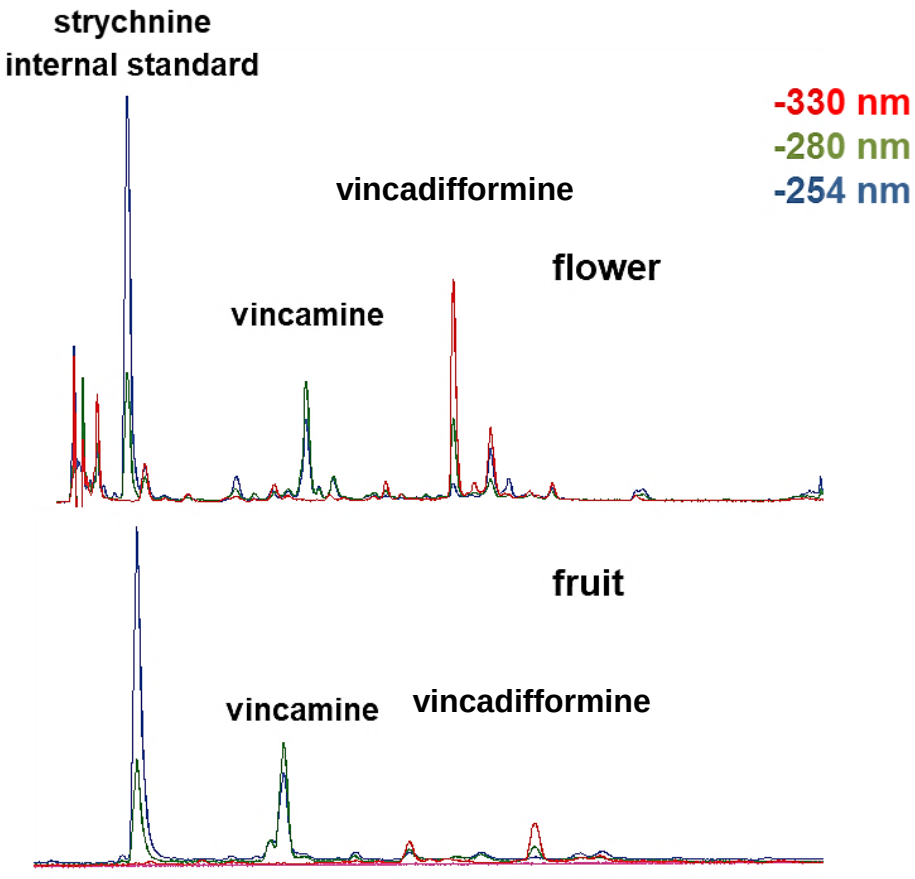


Figure A- 70: HPLC chromatogram of the flowers and fruits of *Vinca minor*.

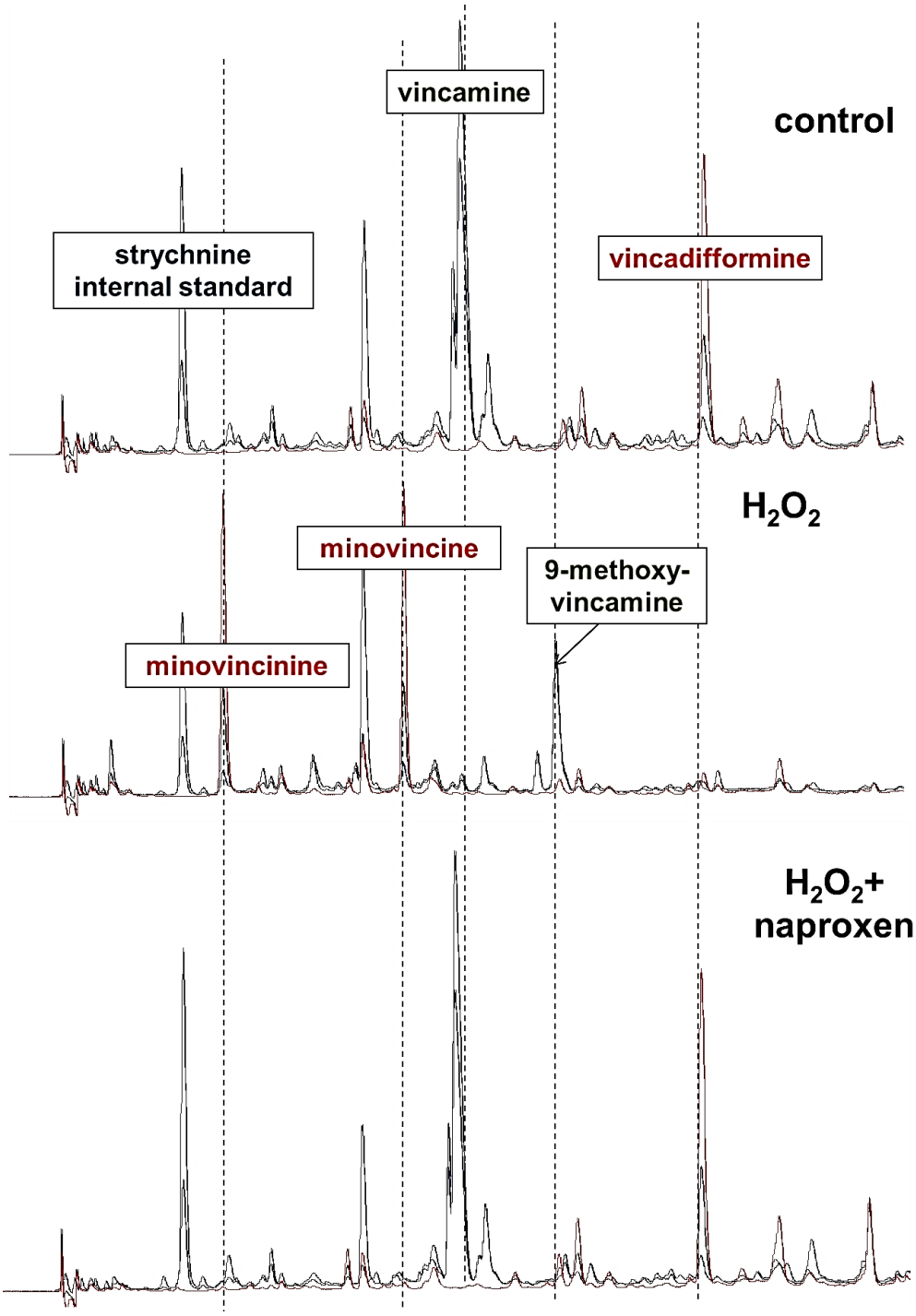


Figure A- 71: Impact of the concomitant application of naproxen and H_2O_2 on the alkaloid spectrum *V. minor*. The plants were sprayed two times during the experiment and collected at 9 days.

Curriculum vitae

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Selmar D., **Abouzeid S.**, Radwan A., Hijazin T., Yahyazadeh M., Nowak M., Lewerenz L. (2018). Horizontal Natural Product Transfer and Allelopathy: Novel insights in a so far unconsidered exchange of natural products. In: Schulz M., Vogt T., Becker C. The Role of Plant Secondary Metabolites in Plant Cross-Kingdom Interactions *Frontiers in Ecology and Evolution, section Chemical Ecology*, Accepted.

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